Characterization of Leptospiral Extracellular Vesicles in Stress Conditions

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การศึกษาลักษณะ Extracellular Vesicles ของเชื้อเลปโตสไปราในการตอบสนองต่อภาวะเครียด

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เชื้อเลปโตสไปราสายพันธุ์ก่อโรคเป็นสาเหตุของโรคเลปโตสไปโรซิส ซึ่งมีการแพร่ระบาดไปทั่วโลกโดยเฉพาะอย่างยิ่งในเขตเมืองและเขตชนบทของประเทศเขตร้อนชื้น พยาธิสภาพของโรคยังไม่เป็นที่ทราบแน่ชัด เชื้อแบคทีเรียสร้างเวสซิเคิลภายนอกเซลล์ (Extracellular vesicles) จากส่วนเมมเบรนของเชื้อโดยบรรจุสารชีวโมเลกุลหลายชนิด ซึ่งอาจมีส่วนเกี่ยวข้องกับระบบขนส่ง การติดต่อสื่อสาร และถูกใช้เป็นวัคซีน เวสซิเคิลภายนอกเซลล์ของเชื้อเลปโตสไปราที่ผลิตภายหลังถูกกระตุ้นด้วยสารเคมีนำมาใช้ในการศึกษาคุณสมบัติและความสามารถในการเป็นวัคซีน แต่ทั้งนี้ยังไม่มีการศึกษาเวสซิเคิลภายนอกเซลล์ชนิดถูกสร้างตามธรรมชาติมาก่อน ดังนั้น ในงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาโปรตีนในเวสซิเคิลภายนอกเซลล์ที่ถูกสร้างตามธรรมชาติจากเซลล์ที่สมบูรณ์ในสภาวะตึงเครียด ได้แก่ การเพิ่มอุณหภูมิเป็น 37 องศาสเซลเซียส และออสโมลาริตีของร่างกาย ซึ่งจำลองการตอบสนองต่อสภาวะที่เชื้อเข้าไปอยู่ในร่างกายขณะเกิดการก่อโรค โดยเปรียบเทียบกับสภาวะที่เลี้ยงในหลอดทดลองที่อุณหภูมิ 30 องศาสเซลเซียส ในการศึกษาเวสซิเคิลภายนอกเซลล์จะถูกคัดแยกและทำบริสุทธิ์โดยใช้หลายวิธีร่วมกัน ได้แก่ การปั่นตกด้วยความเร็วต่ำ การกรอง การปั่นด้วยความเร็วยิ่งยวด และการปั่นตกโดยใช้ความหนาแน่นของน้ำตาลซูโครส พบว่ามีรูปร่างทรงกลมและขนาดนาโนเมตรเมื่อศึกษาภายใต้กล้องจุลทรรศน์อิเล็กตรอนชนิดส่องผ่าน แล้วนำมาระบุชนิดและเปรียบเทียบปริมาณเชิงสัมพัทธ์ของโปรตีนในเวสซิเคิลภายนอกเซลล์จากทั้ง 3 สภาวะโดยวิธีติดฉลากด้วย dimethyl ร่วมกับแมสสเปกโตรเมทรี (LC-MS/MS) จากการศึกษาพบโปรตีนทั้งหมด 690 ชนิด เมื่อทำนายตำแหน่งของโปรตีนด้วยเครื่องมือชีวสารสนเทศ ได้แก่ PSORTb เวอร์ชั่น 3.0.2 CELLO Gneg-mPLoc, SOSUI และ SignalP พบว่ามีโปรตีน 399 ชนิด (ร้อยละ 57.9) อยู่ในไซโตพลาสซึม ตามด้วยโปรตีน 103 ชนิด (ร้อยละ14.8) อยู่ผนังชั้นนอก โปรตีน 101ชนิด (ร้อยละ14.5) อยู่ผนังชั้นใน โปรตีน 36 ชนิด (ร้อยละ5.1) ไม่ทราบตำแหน่ง โปรตีน 27 ชนิด (ร้อยละ4) อยู่ในเพอร์ริพลาสซึม และโปรตีน 24 ชนิด (ร้อยละ3.6) เป็นโปรตีนที่ถูกหลั่งออกนอกเซลล์ นอกจากนี้ ในการทำนายบทบาททางชีวภาพด้วย KEGG pathway ของโปรตีนทั้ง 690 ชนิด พบว่าส่วนใหญ่ไม่ทราบบทบาททางชีวภาพ (ร้อยละ 49.6) ตามด้วยโปรตีนในกระบวนการถอดรหัส (ร้อยละ 10.6) และ เมตาบอลิซึมของคาร์โบไฮเดรต (ร้อยละ 9.1) จากการศึกษาการเปรียบเทียบปริมาณเชิงสัมพัทธ์โปรตีนจากเวสซิเคิลภายนอกเซลล์ที่ถูกสร้างในสภาวะการเพิ่มอุณหภูมิ มีโปรตีน 83 ชนิดที่แสดงออกแตกต่างจากสภาวะที่เลี้ยงในหลอดทดลองอย่างมีนัยสำคัญ (เพิ่มขึ้น 55 ชนิด และลดลง 23 ชนิด) โดย diaminopimelate decarboxylase มีการแสดงออกเพิ่มมากที่สุด 3.9 เท่า ในการเปรียบเทียบปริมาณเชิงสัมพัทธ์ของเวสซิเคิลภายนอกเซลล์ที่ถูกสร้างในสภาวะออสโมลาริตีของร่างกายพบว่ามีโปรตีน 106 ชนิดมีความแตกต่างจากสภาวะห้องปฏิบัติการอย่างมีนัยสำคัญ ซึ่งมีโปรตีนเพิ่มขึ้น 17 ชนิด และลดลง 89 ชนิด โดย transketolase alpha subunit มีการแสดงออกเพิ่มมากที่สุด 1.52 เท่า ทั้งนี้การเปรียบเทียบเชิงสัมพัทธ์ของของเวสซิเคิลภายนอกเซลล์ระหว่างสภาวะออสโมลาริตีของร่างกายและสภาวะเพิ่มอุณหภูมิ พบว่ามีโปรตีน 89 ชนิดที่แสดงออกแตกต่างกันอย่างมีนัยสำคัญ โดยมีโปรตีน sulfate ABC transporter periplasmic sulphate-binding มีการแสดงออกเพิ่มมากที่สุดที่สภาวะเพิ่มอุณหภูมิถึง 2.9 เท่า นอกจากนี้ ยังพบโปรตีนที่ทราบแล้วว่าเป็นปัจจัยก่อโรคหลายชนิด เช่น โปรตีน Lig, LipL21, LipL32, LipL41 และ hemolysin รวมทั้ง โปรตีนที่ยังไม่ทราบหน้าที่อีกหลายชนิด โดยสรุป เวสซิเคิลภายนอกเซลล์ที่ถูกสร้างจากเชื้อเลปโตสไปราในการตอบสนองต่อสภาวะตึงเครียดบรรจุโปรตีนหลากหลายที่แสดงออกแตกต่างกัน ซึ่งอาจมีหน้าที่เกี่ยวข้องกับพยาธิกำเนิดของโรคเลปโตสไปโรซิสและการอยู่รอดของเชื้อเลปโตสไปราในร่างกาย

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Pathogenic *Leptospira* spp. is a causative agent of leptospirosis, a worldwide zoonosis with public health concern especially in the urban slum of metropolis and rural areas in tropical and subtropical countries. The pathogenesis of leptospirosis remains elusive. Extracellular vesicles (ECVs), which pinch off from the bacterial membranes, simultaneously harbor multiple active molecules that may serve as a secretion system, communication tool, and vaccine candidates. Recently, chemically induced leptospiral ECVs were studied and used as vaccine candidates. However, the naturally produced leptospiral ECVs has not been characterized. This study aimed to identify proteins in leptospiral ECVs produced under stress conditions including temperature shift to 37°C and physiologic osmolarity, which mimicked the host environment, in comparison to *in vitro* growth at 30°C. The leptospiral ECVs produced under each condition were isolated and purified from intact cells using combined methods of centrifugation, filtration, ultracentrifugation, and sucrose density gradient centrifugation resulting in nanosized spherical vesicles as shown by transmission electron microscopy. To identify and relatively quantify proteins in these leptospiral ECVs, dimethylation labeling coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed. A total of 690 proteins were identified and predicted their subcellular localization with bioinformatics tools including PSORTb v3.0.2, CELLO, Gneg-mPLoc, SOSUI, and SignalP. Of these, the majority (399 proteins, 57.9%) were predicted as cytoplasmic proteins followed by 103 (14.8%) outer membrane proteins, 101 (14.5%) inner membrane proteins, 36 (5.1%) unknown, 27 (4%) periplasmic proteins, and 24 (3.6%) extracellular proteins. Based on KEGG pathway analysis the identified proteins were biologically categorized into unidentified group (49.6%) followed by transcription (10.6%), and carbohydrate metabolism (9.1%). Relative quantification of protein abundance showed differential expression of proteins cargoes. In response to temperature shift, 83 proteins significantly up- and downregulated (55 and 28, respectively) ($p< 0.05$) of which diaminopimelate decarboxylase was the most up-regulated (3.9-fold). Under physiologic osmolarity, 106 proteins were differentially expressed with 17 up-regulated and 89 down-regulated proteins ($p< 0.05$) of which transketolase alpha subunit protein was the most up-regulated (1.52-fold). In addition, sulfate ABC transporter periplasmic sulphate-binding protein was the most up-regulated (2.9 fold) under temperature shift of all 89 proteins differentially expressed between the stress conditions. Moreover, known virulence factors, such as Lig proteins, LipL21, LipL32, LipL41, and hemolysin, as well as hypothetical proteins were found in leptospiral ECVs. In conclusion, leptospiral ECVs produced in response to stress conditions harboring differentially expressed proteins that may play a role in the pathogenesis of leptospirosis and survival of leptospires in the host.

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LIST OF ABBREVIATIONS

ECVs	Extracellular vesicles
OMVs	Outer membrane vesicles
MVs	Membrane vesicles
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
LPS	Lipopolysaccharide
OMPs	Outer membrane proteins
OM	Outer membrane
IM	Inner membrane
CYT	Cytoplasmic
PM	Periplasmic
UN	Unknown
AUF	Acute undifferentiated fever
PMN	Polymorphonuclear cells
NETs	Neutrophil extracellular traps
MPO	Myeloperoxidase
TEM	Transmission electron microscopy
PAMPs	Pathogen-associated molecules patterns
PRRs	Pathogen recognition receptors

LC-MS/MS	Liquid chromatography tandem mass spectrometry
NTA	Nanoparticles tracking analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
EMJH	Ellinghausen-McCullough-Johnson-Harris
BSA	Bovine serum albumin
PI	Propidium iodide
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WB	Western blotting
HRP	Horseradish peroxidase
IAA	Iodoacetamide
DTT	Dithiothreitol
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline – tween
TNE	Tris sodium chloride EDTA
TEAB	Tris sodium chloride EDTA buffer
TFA	trifluoroacetic acid
FA	Formic acid

CHAPTER I

INTRODUCTION

Bacterial pathogens have several mechanisms to secrete virulence factors to interact with their host cells. The secretion systems are strategies of bacteria to transport individual molecules or small complex molecules to the external environment or directly into host cells (1). Moreover, some gram-negative bacteria produce and release extracellular vesicles (ECVs) during normal growth (2). The ECVs are spherical nano-sized proteolipids of 10-300 nm in diameter which serve as a secretion pathway by which they contain multiple active molecules as cargos, such as proteins, lipopolysaccharides, nucleic acids (DNA and/or RNA), and metabolites (3-6). However, the biogenesis of ECVs is still not well understood. Bacteria release ECVs in response to hostile host environments, such as hydrogen peroxide, sodium chloride, antibiotic, and temperature shift (7-9). ECVs allow virulence factors to reach target cells locally or at a distance and play a role in bacterial pathogenesis. For example, ECVs derived from *Legionella pneumophila* were shown to promote bacterial replication in phagosome of macrophages (10). ECVs of pathogenic *Escherichia coli* were reported to induce cell death in colon epithelial cell line (11). Furthermore, the ECVs have been used as acellular vaccine formulations because

they carry immunogenic cargos, such as lipopolysaccharide (LPS), outer membrane proteins (OMPs), and flagellin (12-14).

Leptospira spp. are slender hook-ended spirochetes and obligate aerobic gram-negative bacteria belonging to the family *Leptospiraceae* (15). Pathogenic *Leptospira* spp. are the causative agents of leptospirosis, a worldwide zoonosis with public health concern especially in the urban slum of metropolis and rural areas in tropical and subtropical countries. Over a million cases of human leptospirosis with more than 60,000 deaths were estimated per year worldwide (16, 17). Humans are accidental hosts and become infected after exposure to the contaminated environment. Patients with leptospirosis present with a broad spectrum of clinical manifestations ranging from mild febrile diseases, such as a headache, fever, loss of appetite, nausea, vomiting, and myalgia, to severe multiorgan involvement, such as pulmonary hemorrhage, myocarditis, aseptic meningitis, hepatic failure, and renal failure (18).

ECVs of pathogenic *Leptospira* were demonstrated and originally called as leptospiral outer membrane vesicles (OMVs) (19). Since the biogenesis of leptospiral ECVs has not been shown to derive only from the outer membrane, it should be more suitable to call this structure in a general term as “extracellular vesicle (ECV)”. Chemically induced leptospiral ECVs prepared by treatment of intact leptospires with

alkaline plasmolysis buffer or citrate buffer were shown by proteomic analysis to contain several OMPs, such as OmpL1, LipL32, LipL36, and LipL41 (19, 20). However, protein components including surface-exposed proteins of the natural ECVs have not been characterized. In addition, the role of ECVs in the pathogenesis of leptospirosis has never been demonstrated. Recently, the hamsters vaccinated with the chemically induced ECVs showed 100% survival protection against the challenge and significant reduction of leptospiral burden in target organs (21). However, protective antigens in leptospiral ECVs have not been characterized.

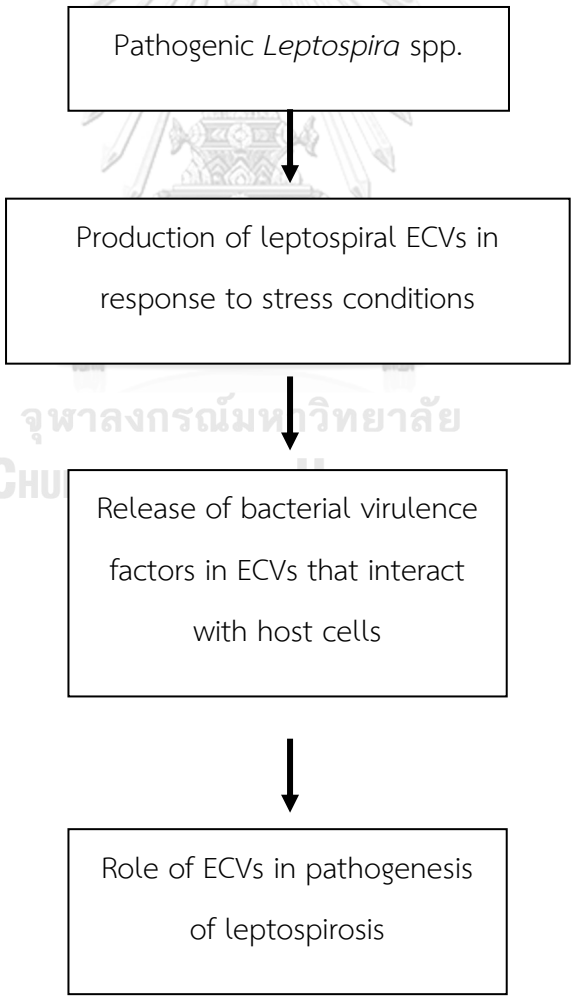
My project aims to characterize leptospiral ECVs produced in response to stress conditions that simulate *in vivo* condition, temperature shift and physiologic osmolarity. The knowledge obtained from this study will demonstrate the potential role of ECVs in the pathogenesis of leptospirosis.

CHAPTER II

OBJECTIVE

To identify proteins in leptospiral ECVs produced under stress conditions including temperature shift to 37°C and physiologic osmolarity, which mimicked the host environment, in comparison to in vitro growth at 30°C.

Conceptual framework



CHAPTER III

REVIEW OF RELATED LITERATURES

Leptospira spp.

Leptospira spp. are obligate aerobic gram-negative bacteria and morphologically characterized as slender hook-ended spirochete with approximately 0.1-0.3 μm in diameter and 6-20 μm in length (22). This genus belongs to the order Spirochetales and family *Leptospiraceae* and comprises both pathogenic and saprophytic species *(*15, 18*).* Based on lipopolysaccharide (LPS) antigens and its sugar composition and structure, *Leptospira* spp. have been serologically classified into more than 250 pathogenic serovars and more than 60 saprophytic serovars *(*18*).* The genetic classification categorized leptospires based on DNA similarity into 35 genospecies including 13 pathogenic species, 11 intermediates species, and 9 non-pathogenic species (23). However, the genetic classification does not correspond to the serological classification. Pathogenic *Leptospira* spp. are the causative agents of leptospirosis, whereas saprophytic *Leptospira* spp. including *L. biflexa* are free living bacteria in the environment *(*15*).* Leptospires can motile using periplasmic endoflagella that are located in the periplasmic space and terminated at each polar end (24). The cell wall of Gram-negative bacteria generally have double membrane

consisting of outer membrane (OM) and inner membrane (IM) or cytoplasmic membrane, and a peptidoglycan layer that is associated with the IM (25). The OM of pathogenic *Leptospira* spp. contains mostly lipopolysaccharide (LPS) on the outer surface (26), lipoproteins, and transmembrane proteins (26). Previously, the spiral shape morphology of leptospires was demonstrated to be contributed by peptidoglycan sacculi and cytoskeletal proteins (27). Genomic analysis reveals that leptospires contain a large and a small chromosome with 3.5-4.2 Mbp and 350 kbp in size, respectively, with GC content of approximately 35-45% (28, 29). The large chromosome called cI mostly encodes housekeeping genes, while the small chromosome called cII encodes genes involved in amino acid biogenesis pathways, such as methylene tetrahydrofolate reductase (*metF*) and aspartate semialdehyde dehydrogenase (*asd*) (30, 31). Leptospires are slow growing gram-negative bacteria under aerobic condition at optimal temperature of 28-30°C in simple enrichment media containing ammonium salt utilized as a nitrogen source and cobalamin (vitamin 12) required in a final step of methionine biogenesis. Due to a lack of hexokinase pathway for sugar utilization, long-chain fatty acid, typically polyoxyethylene sorbitane ester (tween), was utilized as a carbon source by β-oxidative pathway (18, 30). In spite of inability to utilize sugars, leptospires are able to convert glycerol into sugar nucleotides using phosphoglucose isomerase in

gluconeogenesis pathway. Moreover, albumin was supplemented in a serum-free defined culture medium, such as Ellinghausen-McCullough-Johnson-Harris (EMJH), in order to detoxify long chain fatty acid by absorbing and gradual releasing into the culture media (32).

Leptospirosis

Leptospirosis, caused by pathogenic *Leptospira* spp., is recently considered as a re-emerging zoonosis with a worldwide distribution especially in the urban slum of metropolis and rural areas in tropical and subtropical regions (18, 33). Owing to non-specific manifestations diagnosis of leptospirosis was underestimated and inaccurate, especially in developed countries that reported a low number of human leptospirosis, therefore leptospirosis is considered as a neglected disease (34-37). Leptospirosis outbreaks usually increases during rainfall and/or flooding. It is one of important infectious diseases that affect humans with high morbidity and mortality rate with approximately over one million cases of human leptospirosis and 60,000 deaths annually worldwide (16). Moreover, leptospirosis in animals causes economic burden with decreased productivity of livestock and domestic animals, such as pigs, cows, goats, sheep, and dogs, by reducing milk production, abortion, stillbirth, infertility, and death (18, 38). Many occupations and activities, such as farmers, veterinarians, miners, sewage workers, fishermen, cattlemen, and garbage collectors,

are at high risk of exposure to pathogenic leptospires in the environment including water and soil contaminated with urine of reservoir hosts, mostly rodents (39). Human leptospirosis may manifest as a biphasic disease, i.e. containing two phases; the acute or leptospiremic phase in the first week of infection followed by the secondary phase, leptospiruria or immunologic phase within 2 to 4 weeks after infection (40). In the acute phase, patients commonly present with non-specific symptoms, such as a headache, fever, myalgia especially calf pain, loss of appetite, nausea, vomiting, which are difficult to differentiate from other infections including influenza, dengue fever, malaria, and rickettsial infections, therefore leptospirosis is one of clinical syndromes called acute undifferentiated fever (AUF) (40). Clinical manifestations in the immunologic phase are related to target organ involvement, such as renal failure, hepatic failure, pulmonary hemorrhagic syndrome, myocarditis, meningitis, leading to severe leptospirosis, also known as Weil's disease, and death (17, 40-42). Patients with pulmonary hemorrhagic syndrome resulted in approximately 50% mortality (43).

Diagnosis of leptospirosis is quite difficult because of diverse and non-specific manifestations. Therefore, laboratory investigations are required to confirm the diagnosis including direct detection of leptospires in appropriate specimens, such as blood, urine, cerebral spinal fluid, and serological detection of specific antibodies

against leptospiral antigens (41, 44). Technically, selection of specimens and methods for detection of the organism depends on clinical course of disease. During leptospirosis progression, leptospires are usually detected in blood up to 7 days, cerebral spinal fluid during 4-10 days, and urine after 10 days post-infection (45). The methods of leptospiral detection include isolation and culture of leptospires and polymerase chain reaction (PCR) amplification of leptospiral genomic DNA targeting specific targets, such as housekeeping or virulence genes, in appropriate specimens. Specific antibodies against leptospires developed after the second week of infection can be detected by a standard method called microscopic agglutination test (MAT) (18, 46, 47). MAT distinguishes serovar-specific agglutinating antibodies in sera of patients (36). Treatment of leptospirosis is based on severity of the disease (41). Patients with mild symptoms, such as a headache, fever, loss of appetite, nausea, vomiting, may resolve spontaneously or are treated with oral antibiotics, such as doxycycline, while severe cases need intensive care, close observation, appropriate supportive care of target organ involvement, and intravenous antibiotics treatment, such as penicillin and ceftriaxone (40, 41, 48).

Pathogenesis of leptospirosis

Leptospires enter the hosts via cut skin, and mucous membranes of eyes, nose, and throat. Motility of *Leptospira* is necessary for transverse through mucous

layer into the blood vessel and subsequently circulate in the bloodstream, then disseminate to target organs including lung, liver, brain, eyes, and kidney (30). Pathogenic *Leptospira* spp. demonstrated chemotactic behavior to hemoglobin. Pathogenic leptospires express many outer membrane proteins (OMPs) as adhesion molecules on their surface for binding to components on host cells, such as extracellular matrix proteins (collagen, fibronectin, laminin, plasminogen), and host molecules for immune evasion (49-51).

The main mechanism of immune defense against leptospirosis is humoral immunity. To overcome host immune response, pathogenic *Leptospira* spp. express several proteins acting in binding to complement regulators in complement cascades, such as factor H and C4BP in order to evade complement-mediated killing (18, 49). Furthermore, the inhibition of complement cascade reduces not only release of anaphylatoxin (C3a, C4a), which migrate and activate the phagocytic cells, but also opsonins (C3b, C4b) that mediate phagocytosis killing (52). Polymorphonuclear cells (PMN) or neutrophils are also components of innate immune response and play an important role in bacterial infection. Previous studies revealed that neutrophil extracellular traps (NETs) were able to eliminate pathogenic *Leptospira* by bactericidal activity and/or oxidative stress (53, 54). Myeloperoxidase (MPO) is synthesized and packaged in the neutrophil granules which catalyze

hydrogen peroxide (H_2O_2) to incorporate with chloride anions into strong antimicrobial hypochlorous acid (HOCl) (55). Myeloperoxidase (MPO)-containing granules commonly fuse with the phagosome during phagocytosis of neutrophils and are released to extracellular space by which the granules fuse with the cell membrane and involve in degranulation of NETs (56, 57). Pathogenic *Leptospira* express catalase to overcome oxidative stress (58). Recently, LipL21, an OMP of pathogenic *Leptospira*, was shown to inhibit myeloperoxidase activity suggesting that LipL21 might play a critical role in innate immune evasion and survival of pathogenic leptospires in the host (59).

Vaccine in leptospirosis

Leptospirosis vaccines have been developing for centuries. After the discovery pathogenic *Leptospira* spp. in 1913, within a year later the first vaccine was prepared (60). Bacterin, heat-killed inactivated whole cell vaccine, is the first type of leptospirosis vaccine. Experimental immunization to susceptible host revealed that bacterins provide protection to leptospires (60, 61). Subsequently, live attenuated vaccines and LPS vaccines were developed and tested in animal models (18, 62). Although bacterins provide protection against leptospirosis, there are several limitations including reactogenicity, short-term protection requiring booster doses every 1-2 years, and serovar restriction (18, 63, 64). Recently, experimental

immunization to susceptible hosts with LPS-biosynthesis mutant strains showed the absence of clinical signs of infection after challenge with not only homologous strain but also heterologous strains (64), suggesting that the antigens eliciting protective immune responses are non-LPS antigens, possibly conserved protein antigens. Recombinant proteins used as subunit vaccines might act as T-dependent antigens that induce antibody production and memory immune response resulting in long-term protection (64). The outer membrane proteins (OMPs), highly conserved among pathogenic leptospires, have been used as vaccine candidates (65). So far potential vaccine candidates have been identified by several approaches, such as high-throughput screening of surface-exposed proteins and beta-barrel transmembrane proteins (65, 66). Immunization with recombinant OMPs, such as OmpL37, LigA, LigBม and LipL32 induced partial protective immunity against leptospirosis in susceptible animal models (64, 67-69). However, no subunit vaccines have currently induced complete protection against leptospirosis.

Extracellular vesicles

Extracellular vesicles (ECVs), also called outer membrane vesicles (OMVs) and membrane vesicles (MVs), are spherical nano-sized proteolipid particles of 10-300 nm in diameter (7) The production and secretion of ECVs is found in gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*,

Porphyromonas gingivalis, and *Leptospira interrogans* (8, 11, 20, 70-72). ECVs harbor active multi-molecules, such as proteins, lipopolysaccharide (LPS), nucleic acids (DNA and/or RNA), and metabolites produced during normal bacterial growth (4, 5, 73). Previous studies showed biological properties of ECVs in the cell to cell contact locally or at a distance and play a role in bacterial pathogenesis, such as intracellular communication, biofilm formation, promoting bacterial replication, inducing host cell injury, and evasion of immune response (6, 10, 70, 71, 74). ECVs derived from pathogenic *E. coli* carried multiple virulence factors, such as shiga toxin 2a (Stx2a) which induced cell death in colon epithelial cell (11). *Bacteroides fragilis* ECVs delivered mature toxins to target cells and caused cell injury (3). *Legionella pneumophila* ECVs altered macrophages to promote bacterial survival inside the cells (10). In addition, a previous study on the ulcer biopsy from patients infected with *Helicobacter pylori* revealed membrane-bound vesicles by transmission electron microscopy (TEM) (75). The membrane bound structures were shown to contain outer membrane protein VacA by immunoblotting.

Currently, the mechanism of ECV biogenesis is still unclear. Bacteria encountering environmental stress increased ECV production as the bacterial adaptive response by rapid release of outer membrane compartment to promote bacterial survival (76). Previous reports showed that several environmental stresses

including temperature shift, osmolarity change, oxidative agents, and antibiotic exposure were able to trigger ECV production (8, 9). The mimic conditions of host milieu, such as temperature shift from 25°C to 37°C, the addition of 2 M sodium chloride (NaCl), and exposure to 250 μM H_2O_2, were shown to increase ECV production in pathogenic *P. aeruginosa* (8). *Pseudomonas putida* released ECVs in higher yield after exposure to NaCl, EDTA, as well as temperature shift (9). ECVs may be generated by pinching off bacterial outer membrane leaflet as natural ECVs and therefore consist of multiple immunogenic molecules including outer membrane proteins (OMPs), LPS, flagellin, and other naïve conformational antigens. These components can act as pathogen-associated molecular patterns (PAMPs) that interact with host pathogen recognition receptors (PRRs) to stimulate innate immune response, and subsequently promote adaptive immunity against pathogens (13, 14). Therefore, ECVs have been used as acellular vaccine formulations against some bacterial infections (12, 77) including leptospirosis (21).

Leptospiral extracellular vesicles

To characterize leptospiral OMPs, outer membrane vesicles (OMVs) were induced by treating *Leptospira kirchneri* with alkaline plasmolysis buffer pH 9.0, isolated by sucrose gradient ultracentrifugation, and detected by transmission electron microscopy (TEM) (20). Immunoblotting demonstrated that the isolated

OMVs contained OMPs, such as OmpL1, LipL21, LipL32, LipL36, and LipL45. In addition, chemically induced OMVs of *Leptospira* were also prepared by incubating intact leptospires with citrate buffer pH 3.0 and isolated by sucrose gradient ultracentrifugation (19). Proteomic analysis of the isolated OMV fractions by liquid chromatography tandem mass spectrometry (LC-MS/MS) showed additional OMPs including hypothetical proteins and annotated proteins, such as Loa22, OmpA, LipL41, glycosyl hydrolase, serine protease and flagellar hook-associated protein (19). *Leptospira* mutant strain with the inactivated gene encoding HtpX-like M48 metalloprotease, a bacterial enzyme responding to stress and homeostasis, showed bacterial membrane instability and increased OMV production (78). When the mutant strain exposed to 10-fold increase of iron concentration, OMVs were released to precipitate the extreme iron. This response may help promote bacterial survival.

Applications of extracellular vesicles

Extracellular vesicles are now widely recognized as one of bacterial secretion systems in both gram-negative and gram positive bacteria (79, 80). Owing to biologically multi-active molecules in ECVs, bacteria utilize the ECVs in multifaceted functions including communication, competition, nutrient acquisition, and pathogenesis association (Table 1) (81). Recent studies reported the ECVs elicit immune response (82). Immunostimulatory ligands known as pathogen-associated

molecular patterns (PAMPs), such as LPS, lipoprotein, nucleic acid and peptidoglycan are normally harbored with ECVs, are recognized by receptor on surface of epithelia cell and innate cell known as pathogen recognition receptors (PRRs). PAMPs are processed and presented to adaptive immune cells and then the humoral immune response is induced. Due to immunogenic properties, ECVs derived from several bacteria have been used as a vaccine platform. Multivalent conjugate vaccines have been developed for *Neisseria meningitidis*, a main cause of meningitis and septicemia (83, 84). However, these expensive vaccines limit their use in the high incidence areas (85). The OMVs of *N. meningitidis* were investigated as acellular vaccines that showed immunogenicity and induced protective immunity determined by serum bactericidal assay (85). Acellular pertussis vaccine against *Bordetella pertussis,* a causative agent of pertussis, provides partial protection against the current circulating strains (86, 87). Then, the OMVs derived from *B. pertussis* used as a vaccine candidate resulted in decreased bacterial burden in the target organ (lung) and accumulated tissue-resident memory CD4+ T cells that play a critical role in sustained protective immunity against *B. pertussis* (88-90). Recently, the chemically induced OMVs of pathogenic *Leptospira* spp. were evaluated as acellular vaccine formulations in a hamster model and showed 100% survival protection with significantly decreased

bacterial burden in target organs including liver, lung, and kidney (21). Nevertheless, the key protective antigens in the OMVs have not been identified.

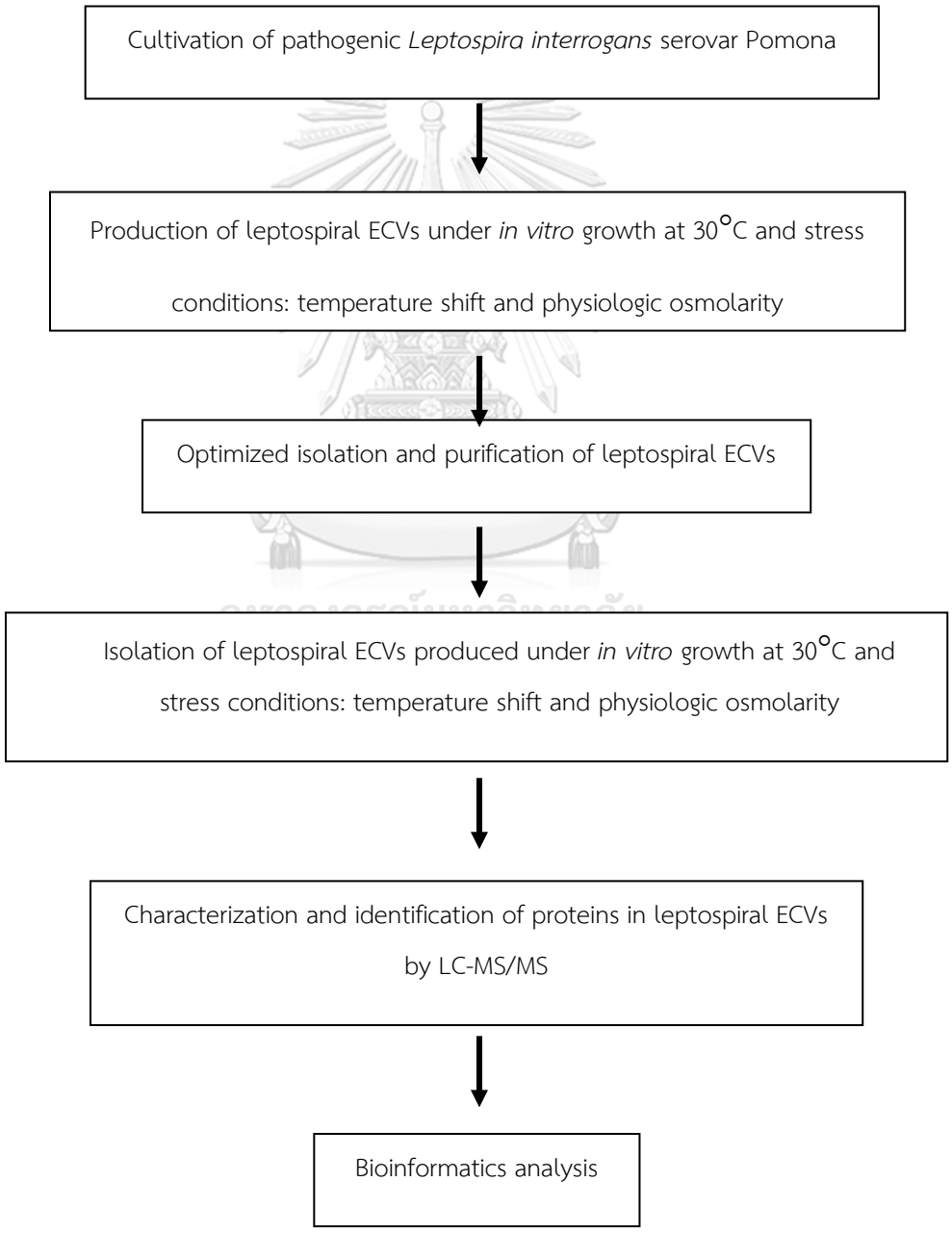
Table 1 Bacterial extracellular vesicles containing virulence factors and their associated functions (81)

S. No	Bacterial species	Virulence factors as OMV component	Associated function	Reference
1	*Escherichia coli* [Enterotoxigenic *E. coli* (ETEC), Shiga toxin producing *E. coli* (STEC), Enterohemorrhagic *E. coli* (EHEC)]	Heat labile enterotoxin (LT), Shiga toxin, Cytolysin A (ClyA)	Pore forming ability, enterotoxic and vacuolating activity, cytotoxicity	Kolling and Matthews, 1999; Horstman and Kuehn, 2000; Yokoyama et al., 2000; Wai et al., 2003; Kuehn and Kesty, 2005; Kwon et al., 2009; Mendez et al., 2012; Jun et al., 2013
2	*Helicobacter pylori*	Vacuolating toxin (VacA), Lewis antigen LPS, Helicobacter cysteine rich proteins (Hcp), Sialic acid binding adhesion (SabA)	Adherence, cytotoxic and vacuolating activity, cell proliferation activity	Fiocca et al., 1999; Keenan et al., 2000; Mullaney et al., 2009; Olofsson et al., 2010; Jun et al., 2013
3	*Pseudomonas aeruginosa*	Alkaline phosphatase, Phospholipase C Protease, Hemolysin, Pseudomonas quinolone signal (PQS), Cif, hydrolases	*In vitro* enzyme activities, cytokine stimulation, bactericidal quinolines	Kadurugamuwa and Beveridge, 1995, 1996; Li et al., 1998; Mashburn and Whiteley, 2005; Mashburn-Warren et al., 2008; Bomberger et al., 2009; Ellis et al., 2010; Choi et al., 2011; Toyofuku et al., 2012
4	*Borrelia burgdorferi*	Outer surface proteins (OspA, B, D)	Adherence to host cells	Dorward et al., 1991; Shoberg and Thomas, 1993, 1995
5	*Shigella flexneri*	Invasion plasmid antigens (IpaB, C,D)	Invasion of host tissue	Kadurugamuwa and Beveridge, 1998
6	*Shigella dysenteriae*	Shiga toxin (Stx)	Cytotoxicity, host cell apoptosis	Dutta et al., 2004
7	*Salmonella typhi*	Outer membrane protein (OmpC), ClyA	Pore forming activity	Bergman et al., 2005
8	*Treponema denticola*	Proteases, Dentilisin	Chymotryptic activity, disruption of tight junctions	Rosen et al., 1995; Chi et al., 2003
9	*Neisseria meningitis*	NarE, NlpB, PorA, B	Cytokine production, fibrinolytic activity, adherence to host cells	Ferrari et al., 2006; Vipond et al., 2006; Massari et al., 2010; Van De Waterbeemd et al., 2013
10	*Bordetella pertussis*	Pertussis toxin (Ptx), Adenylate cyclase hemolysin	Cytotoxicity	Hozbor et al., 1999
11	*Burkholderia cepacia*	Phospholipas-N, Hemagglutinin	Enzyme activities	Allan et al., 2003
12	*Vibrio cholera*	Rtx toxin, LPS	Depolymerising actin, stimulatory response	Bishop et al., 2010; Altindis et al., 2014
13	*Xanthomonas campestris*	Type-3 secretion proteins, cellulase, xylosidae	Enzyme activity, insecticidal activity	Sidhu et al., 2008
14	*Legionella pneumophila*	Acid phosphatase (Map), Protease (Msp), Chitinase (ChiA), Hsp60	Adherence to ECM, enzyme activity	Fernandez-Moreira et al., 2006; Galka et al., 2008
15	*Moraxella catarrhalis*	Ubiquitous surface protein (UspA1, A2)	Complement binding	Tan et al., 2007; Vidakovics et al., 2010
16	*Acinetobacter baumannii*	Outer membrane protein (AbOmpA), PAMPS (LPS, flagellin),Proteases, Phospholipases, SOD, Catalase	Binding to host tissues, Immunomodulatory effect, enzyme activity	Kwon et al., 2009; Mendez et al., 2012; Moon et al., 2012; Jun et al., 2013
17	*Campylobacter jejuni*	Cytolethal distending toxin (CDT)	Adhesion and invasion, immunomodulatory effect	Elmi et al., 2012; Jang et al., 2014
18	*Porphyromonas gingivalis*	CTD family proteins such as gingipains (RgpA, RgpB, Kgp)	Adherence, host tissue Invasion, immune evasion	Veith et al., 2014
19	*Yersinia pestis*	Adhesin Ail, Protease Pla, F1 outer fimbrial antigen	Complement binding, enzyme activity	Eddy et al., 2014
20	*Cronobacter sp. [C. sakazakii, C. turicensis, C. malonaticus]*	Outer membrane protein (OmpA and OmpX)	Binding to host cell receptors	Kothary et al., 2017

CHAPTER IV

MATERIALS AND METHODS

Research design



Leptospiral cultivation

Low passage *Leptospira interrogans* serovar Pomona (kindly provided from Professor Ben Adler, Monash University, Australia and were maintained at the faculty of Medicine, Khon Kaen University, Thailand) were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) broth (Difco™ Leptospira Medium Base EMJH) contained 10% albumin fatty acid at 28-30°C until cells density of approximately 10^8 cells/mL was reached representing the exponential growth phase. The cells density of leptospires was determined by counting cells using Petroff-Hauser counting chamber (Hauser Scientific) under dark-field microscopy (91). The cell viability was determined by fluorescent staining using Live/Dead BactLight Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific, Produce No. l7007).

Culture at stress conditions

Physiologic osmolarity. To mimic physiologic osmolarity, approximately 10^8 cells/ml of leptospires in EMJH were supplemented with 120 mM sodium chloride as previously described (92) and then incubated at 30°C overnight. The cell viability was determined by fluorescent staining using Live/Dead BactLight Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific, Produce No. l7007). The intact cells were removed by centrifugation and filtration, respectively. The supernatant was further subjected to isolation and purification of leptospiral ECVs.

Temperature shift .Leptospires at the exponential phase (approximately 10^8 cells/ml) initially grown at 30 ℃ were then incubated at 37℃ overnight (93). The cell viability was determined by fluorescent staining using Live/Dead BactLight Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific, Produce No. l7007). The intact cells were removed, and the supernatant was further subjected to isolation and purification of leptospiral ECVs

Live/Dead fluorescent viability staining

Propidium iodine (PI) and SYTO9 in Live/Dead BactLight Bacterial Viability Kit were added to leptospires samples at the ratio of 1:1:100 of SYTO9, PI, and leptospires sample, respectively, followed by gentle mixing and standing on ice in the dark for 15 min according to manufacturer's instruction. For a control of dead cells, 90 ul of leptospires (10^8 cells/ml) were treated with 10 ul of 99.8% cold methanol followed by mixing with 1 ul of each dye and standing on ice in the dark for 15 min. The treated samples were observed under fluorescence microscopy at the excitation/emission wavelength of 490/635 nm for propidium iodine and 480/500 nm for SYTO9. All samples were performed in replicate.

Isolation and purification of leptospiral ECVs

Optimization of centrifugation force. In order to remove intact cells and maintain the cell viability, various gravity forces and centrifugation periods were verified. The exponential-phase leptospires were centrifuged at 3,000×g, 9,000×g and 15,000×g for 15 min. (93, 94) Subsequently, the pellets were resuspended in EMJH media without bovine serum albumin (BSA) followed by determining the cell viability using Live/Dead BactLight Bacterial Viability Kit**.**

Isolation of leptospiral ECVs. After removing intact cell, the supernatant was collected and filtered through 0.45 μm and 0.22 μm pore size nitrocellulose filter membrane (Merck Millipore, Ireland), respectively. The filtered supernatant was subject to ultracentrifugation at 200,000×g at 4°C for 1 h using ultra-clear polycarbonate ultracentrifuge tube (Backman Coulter, USA), and Ti 45 type rotor (Beckman Coulter, USA) (95). The pellets were collected and resuspended in EMJH media without BSA for further purification.

Purification of leptospiral ECVs. To purify leptospiral ECVs, the sucrose density gradient centrifugation at the concentration of 5% stepwise increase of 20-60% sucrose (w/v) in Tris sodium chloride buffer (TNE buffer) was used to purify leptospiral ECV as previously described (21). The density sucrose gradient was prepared in the polypropylene centrifuge tube (Beckman Coulter, USA) by gentle

pipetting down 800 µl of sucrose at each concentration with 5% concentration interval from 60% to 20% (bottom to top), respectively. The previously ultracentrifuged preparation of leptospiral ECVs was added at the top (the lightest fraction) of sucrose gradient followed by centrifugation at 77,000×g at 4 ℃ for 16-18 h. Subsequently, 800 µL of each sucrose fraction was gently removed, and protein concentration was measured by Micro BCA™ Protein Assay Kit (Thermo Scientific™ BCA Protein Assay Kit Product No. 23235) according to the manufacturer's instruction.

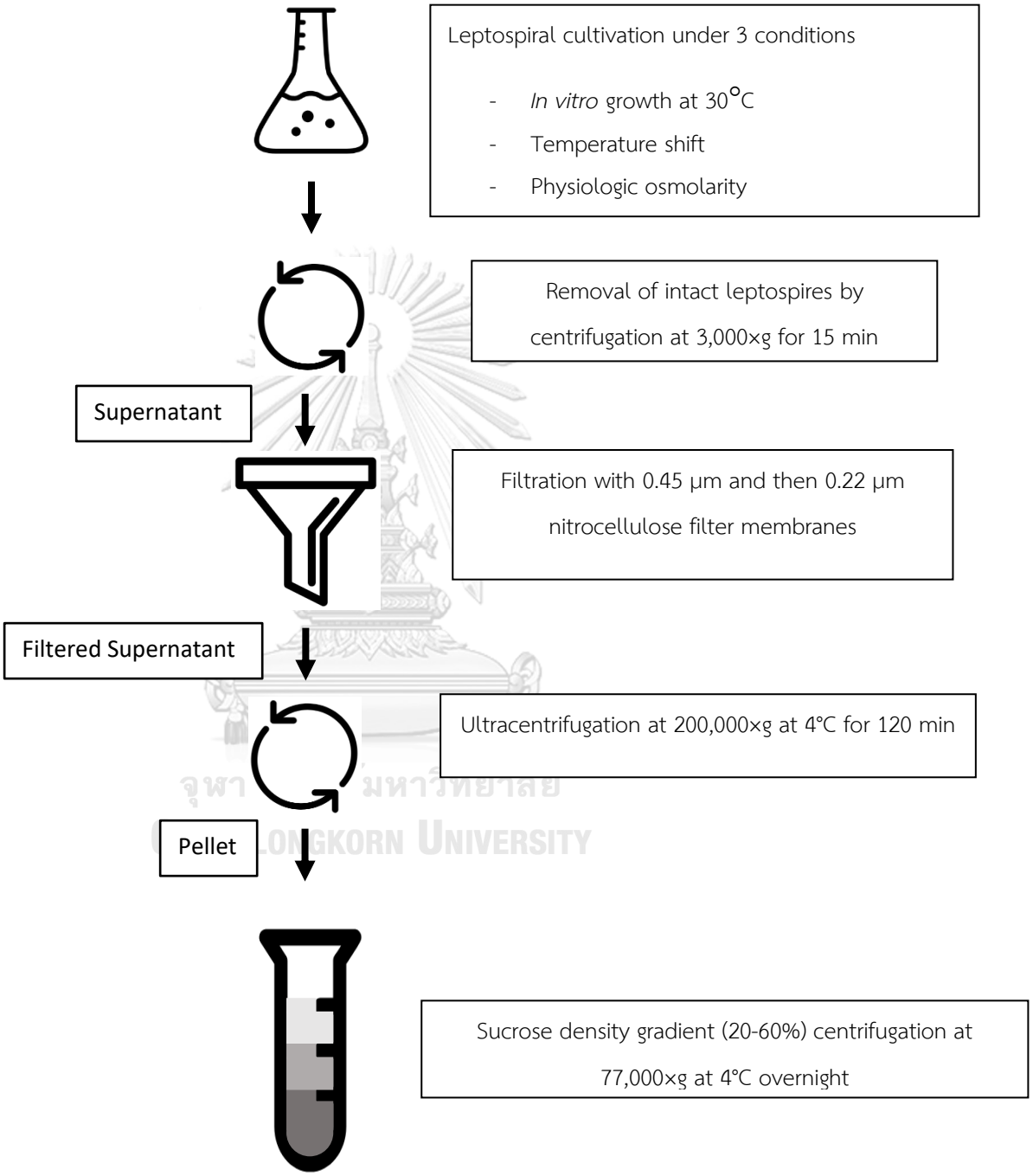


Figure 1. Flow chart showing the steps of isolation and purification of leptospiral extracellular vesicles

Measurement of protein concentration

Micro BCA working reagent (WR) was prepared as the manufacturer's instruction (Thermo Scientific™ BCA Protein Assay Kit Product No. 23235). Micro BCA WR was prepared by mixing 25 parts of Micro BCA reagent A (MA) and 24 parts of Micro BCA reagent B (MB) and 1 part of Micro BCA reagent C (MC). The standard solution (2mg/ml bovine serum albumin, BSA stock) was diluted with 2 M urea in 100 mM triethylammonium bicarbonate (TEAB) buffer (Sigma, Cat No. T7408) to a concentration range of 0-200 µg/ml. The protein samples were diluted with 100 mM TEAB buffer to a final concentration of 2 M urea.(diluted lysis buffer with compatible for Micro BCA reaction) 150 µl of each standard and sample were pipetted into 96 flatted-bottom microwell plates (Thermo Fisher scientific, Denmark), and then 150 µl of WR was added and gently mixed. The microwell plate was covered with Sealing Trap (Thermo Scientific™ Sealing Tape for 96-Well Plates Product No. 15041). To homogeneously mix, the microwell plate was thoroughly shaken on a plate shaker for 30 sec and incubated at 37°C for 2 h. After incubation, the microwell plate was cool downed at room temperature. The elucidation of protein concentration was carried out with measurement of colorimetric absorbance at 562 nm on a plate reader (Thermo Scientific™ Varioskan™ Flash Multimode Reader Product Code: MIB#5250030). All samples were performed in duplicate. The absorbance of replicate

samples were averaged and then subtracted with that of the blank standard (buffer without protein). The standard curve was plotted with the subtracted absorbance against its concentration. Protein concentrations were determined by comparing to the standard curve.

The size distribution of the ECVs

To assess size distribution of leptospiral ECVs in sucrose gradient fractions, the dynamic light scattering was employed using Nano Sight NS300 (Malvern Instruments, United Kingdom) and the results were analyzed with NanoSight Software NTA. The sucrose fraction samples were 50 times diluted with distilled water. Each diluted sample was then injected into Low Volume Flow Cell chamber (LVFC) using disposable syringe at a speed of 0.05 ml per second. Each sample was analyzed in triplicate (72).

Transmission electron microscopy

The morphological characterization of leptospiral ECVs was performed using negative staining by UranyLess EM Stain as the manufacturer's instruction. The sample was gently dropped on Formvar- carbon coated grids and absorbed for 1 min at room temperature, and excess of sample was removed by careful blotting off with a filter paper. Next, the grid was washed with distilled water once and then transferred to a 10 µl of UranyLess (Delta Microscopies, France) for 1 min at room

temperature. The grid was then removed excess negative staining solution with blotting using a filter paper. The grid was dried at least 5 min in a desiccator. The grid was viewed in JEM 1400 transmission electron microscopy (Faculty of Medicine, Chulalongkorn University)

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and Western blotting

The sample were added with a final concentration of 1x SDS sample buffer followed by heating at 100°C for 15 min. Each sample was loaded at 10 µg into the well of 15% sodium dodecyl polyacrylamide gel assembled in an electrophoresis running system under 1x running buffer. The running system was carried out under an electric field at 120 accelerating voltage for 90 min. The proteins were detected by staining the gel with Coomassie brilliant blue R-250 (Bio-Rad, USA) for 30 min followed by destaining with a destaining solution until the background was completely cleared.

The separated proteins in the gel were transferred onto a 0.45 pore size nitrocellulose membrane (Bio-Rad, USA) with a semi-dry transfer system (SemiDry Transblot,Bio-Rad) at 15 accelerating voltage for 45 min using a blotting buffer. The transferred membrane was blocked with phosphate buffer saline containing 0.05% Tween 20 (PBST) and 1% BSA for at least 60 min followed by washing twice with

PBST without BSA. After blocking, the membrane was incubated with a primary antibody: anti-LipL32 mouse monoclonal antibody (1:10,000) in 1% BSA in PBST for 60 min at room temperature and washed with PBST for 15 min. Then the membrane was incubated with a secondary antibody: goat anti-mouse IgG conjugated with HRP (Horseradish peroxidase) (1:20,000) in PBST with 1% BSA for 60 min at room temperature followed by washing with PBST without BSA for 15 min. Subsequently, the immunoblotted membrane was stained with a chemiluminescent HRP substrate (Western blotting detection reagent, Amersham™ ECL™ Prime, RPN22323, GE Healthcare) and detected by chemiluminescence detection system (ChemDoc, BioRad).

In-solution digestion

The in-solution digestion was performed as previous described (96) with slight modification. The samples were buffer exchanged with 8 M urea using 10 kDa Amicon® Ultra 0.5 ml centrifugal filters (Merck Millipore, Ireland). The columns were equilibrated with 8 M urea in 100 mM TEAB buffer and centrifuged at 14,000×g at 4°C for 30 min. The samples were subsequently added into the column and centrifuged at 14,000×g at 4°C for 30 min. the samples were washed twice with 8 M urea in 100 mM TEAB buffer. Then the samples were adjusted the volume to 100 µl. The samples were treated with a reducing agent, 10 mM final concentration of

dithiothreitol (DTT), for 30 min at 37°C with 300 rpm shaking followed by alkylation with 40 mM final concentration of iodoacetamide (IAA) in the dark at room temperature for 30 min. The reaction was quenched by incubation with 10 mM final concentration of DTT at room temperature for at least 15 min. After reduction and alkylation, the samples were enzymatically digested into peptides with porcine trypsin (Thermo Fisher scientific, Cat No. 90058) at a ratio of 1:50 (w/w) at 37°C overnight (no longer than 16 h). To stop the digestion reaction, the samples were transferred on ice and then completely dried with a speed vacuum concentrator.

Measurement of peptide concentration

The peptide concentration was assessed by Quantitative Fluorometric Peptide Assay (Thermo Scientific, Product no. 23290) as manufacturer's instruction. The mixture of peptides generated from trypsin digestion was used as a standard at a working dilution ranging 0-1000 µg/ml in 100mM TEAB buffer. 10 µl of each standard and sample was added into the wells of the fluorescent compatible microplate (Thermo Scientific™ 96-well Black Plates, Product no. 88378). Subsequently, 70 µl of fluorometric peptide assay buffer was added into the wells containing standard and samples followed by adding 20 µl of fluorometric peptide assay reagent. The plate was covered by sealing trap plate (Thermo Scientific™ Sealing Tape for 96-Well Plates Product No. 15036) and incubated at room temperature for 5 min. The fluorometric

measurement of peptides was carried out at the excitation/emission wavelength at 390/475 nm on a plate reader (Fisher Scientific™ BioTek Cytation 5 imaging reader, USA). The raw data were analyzed using Gen5 version 3.04. The standard curve was plotted and used to determine the peptide concentration of each sample.

Dimethyl labeling for mass spectrometry

The enzymatically digested samples were completely reconstituted with 100 µl of 100 mM TEAB buffer. 15 µl of 4% (v/v) formaldehyde isotope was added to individual sample for the light (CH_2O), intermediate (CD_2O) and heavy ($^{13}CD_2O$) labeling followed by mixing briefly and spinning down. Then, the samples were added with 30 µl of 0.6 M sodium cyanoborohydride ($NaBH_3CN$) for light and intermediate labeling and added with 30 µl of 0.6 M NaD_3CN for heavy labeling followed by incubation at room temperature and shaking for 1 h. To quench the labeling reaction, 30 µl of 1% (v/v) ammonium solution was added into individual sample on ice followed by adding 15 µl of formic acid to further quenching the reaction. Subsequently, the labeled samples were pooled and completely dried with a speed vacuum concentrator. Each sample was perform in six biological replicate.

Acetone precipitation

The leptospires were treated with cell lysis buffer containing 2% SDS in 100mM TEAB followed by sonication with aptitude output 35% for 10-sec pulse and

5-sec rest for 5 min. 300 µg of protein was taken into a new tube and was adjusted to a final volume of 500 µl with 100 mM TEAB. Next, the protein was treated with a reducing reagent, 100mM final concentration of DTT, followed by incubation at 37℃ for 30 min with 300 rpm shaking, and alkylation with 40 mM final concentration of iodoacetamide (IAA) in the dark at room temperature for 30 min. The reaction was quenched with 10 mM final concentration of DTT at room temperature for 15 min. Six volume of cold acetone was added to the protein followed by overnight incubation at −20℃. After incubation, the protein was centrifuged at 12,000 ×g at 4 ℃ for 10 min. The pellet was completely reconstituted with 0.6 M final concentration of urea followed by sonication with aptitude output 35% for 10-sec pulse and 5-sec rest for 10 min. Subsequently, the protein was enzymatically digested with porcine trypsin (1:50 ratio) and incubated at 37℃ for 16 h. The peptide concentration was measured with quantitative fluorometric peptide assay. 100 µg of the peptide was taken to a new tube and completely dried with a speed vacuum concentrator.

Peptide fractionation for mass spectrometry

The peptide fractionation was carried out by using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific™, Product No. 84868) as manufacturer's instruction. The dried pellet of peptides was reconstituted with 0.1%

trifluoroacetic acid (TFA) in distilled water to a final volume of 300 µl. The elution solution containing acetonitrile and 0.1% triethylamine was prepared in 10 dilutions from 5% to 50% in 0.1% triethylamine. To perform peptide fractionation, the protective white tip at the bottom of fractionation column was removed and the column was placed to a 2 ml tube followed by centrifugation at 5,000×g for 2 min and then discarded the solution from the tube. Next, the column was equilibrated with 300 µl of 100% acetonitrile followed by centrifugation twice at 5,000×g for 2 min. The column was washed twice with 0.1% TFA solution. Then, 300 µl of peptide sample was added followed by centrifugation at 3,000×g for 2 min. The solution was collected as a flow-through fraction. Next, the column was placed into a new 2 ml tube and 300 µl of type I water was added onto the column followed by centrifugation at 3,000×g for 2 min. The solution was collected as a wash fraction. Next, the column was placed into a new 2 ml tube and loaded with 300 µl of the elution solution (5% acetonitrile in 0.1% TFA) followed by centrifugation at 3,000×g for 2 min. Then, the elution solution step was repeated with increasing percentage of acetonitrile in 0.1% TFA and the solution in the collection tube was retained as a peptide fraction. Subsequently, the peptide fractions were completely dried with a speed vacuum concentrator.

Data processing for analysis of relative protein abundance

Peptide mixtures were analyzed by LC/ MS/MS using an EASY-nLC1000 system coupled to a Q-Exactive Orbitrap Plus mass spectrometer equipped with a nano-electrospray ion source (Thermo Scientific, San Jose, CA). The 5 µl (300 ng) of the peptide mixture was injected into EASY-Spray PepMap RSLC C18 Column (Thermo Fisher Scientific, 2um,100 Å, 50 um x 25 cm) C18 nanoAcquity UPLC trap column (Waters, 0.18 × 20 mm, 5 µm, 100 Å) with a flow rate of 300 nl/min and separated on a BEH300C18 nanoAcquity UPLC column (Waters, 0.075 × 250 mm, 1.7 µm, 300 Å) using a linear gradient of 5–40% of solvent B in 80 min. The peptides were eluted with 5-20% acetonitrile containing 0.1% FA for 43 min followed by 40-98% acetonitrile containing 0.1% FA for 10 min at a flow rate of 300 nl/min. The full scan measured in the Orbitrap mass analyzer at a mass resolution of 70,000 were followed by 10 data dependent MS2 scans at a resolution of 17500. The normalized collision energy of higher-energy collision dissociation (HCD) fragmentation was set at 27. An MS scan range of 400-1600 m/z were selected and monoisotopic precursor ion with unassigned charge states, a charge state of +1 or a charge state of greater than +8 were excluded. Dynamic exclusion was set for 30s was used. Peak list generating software used was Thermo Xcalibur 3.0.63.3. Mass spectra data from LC-MS/MS were matched with peptide sequences by Andromeda software. The MS raw

data (MGF file) were advance searched by the Global Proteome Machine (GPM) database of prokaryotes bacteria *Leptospira interrogans* serovar Copenhageni Fiocruz L1130 containing the forward and reversed peptide sequences. The parameter was set as follow: For measurement errors, fragment mass error was 10 ppm, parent mass error was ±10 ppm, fragment type was monoisotopic. The search parameter included trypsin as the proteolytic enzyme with 1 missed cleavage with Cleavage C-terminal change +17.002735 Da and Cleavage N-terminal change +1.007825 Da. The identifier number (GI number) were uploaded tp retrieve the FASTA file in UniprotKB (http://www.uniprot.org/uploadlists/). FASTA files were exported to bioinformatic localization tools including PSORTb v.3.0.2 (https://www.psort.org/psortb), CELLO (http://cello.life.nctu.edu.tw/), Gneg-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gneg-multi/), SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuigramn/sosuigramn_submit.html) and SignalP (http://www.cbs.dtu.dk/services/SignalP-4.1/) to predict subcellular localization of gram-negative bacteria. Moreover, the identified proteins were biologically categorized their function using KEGG pathway (https://www.uniprot.org/).

Statistical analysis of relative protein abundance

To relatively quantify protein abundance, the identified proteins (found ≥3 out of 6 replicates) of leptospiral ECVs under three conditions were log10

transformation. The log transformed values of identified proteins that belongs to the same protein were averaged. The resulting protein expression data were analyzed across the three conditions with paring including temperature shift vs *in vitro* growth at 30°C, physiologic osmolarity vs *in vitro* growth at 30°C, and physiologic osmolarity vs temperature shift using *t*-tests that were employed to test the differential expression of proteins in leptospiral ECVs under three conditions. This analysis corresponded to a False Discovery Rate (FDR) of < 1%. The expression of differentially expressed proteins of leptospiral ECVs under three conditions were visualized as a volcano plot using Instant Clue.

CHAPTER V

RESULTS

Evaluation of intact leptospires after exposure to stress conditions

Pathogenic *L. interrogans* serovar Pomona were initially grown in EMJH medium with 10% BSA at 30°C (*in vitro* grown) and then were exposed to *in vivo*-simulated stress conditions including temperature shift to 37°C or physiologic osmolarity (EMJH medium supplemented with 120 mM NaCl). To confirm that ECVs were produced naturally from intact cells, leptospires were evaluated by Live/Dead BactLight Bacterial Viability Kit generally used to determine cell viability based on membrane integrity. Cells stained with SYTO9 in green and propidium iodide (PI) in red represented viable (intact) and dead (non-intact) cells, respectively. The results indicated that most *in vitro* cultivated leptospires 96.68% were intact (Fig. 2A). Most leptospires under temperature shift 90.57% and physiologic osmolarity 95.28% remained intact (Fig. 2B and 2C, respectively) as well. Cells treated with cooled methanol used as a control of dead (non-intact) cells were stained with PI (red) as expected (Fig. 2D). The results indicated that most leptospires remained intact under the stress conditions and then were used for isolation of natural ECVs.

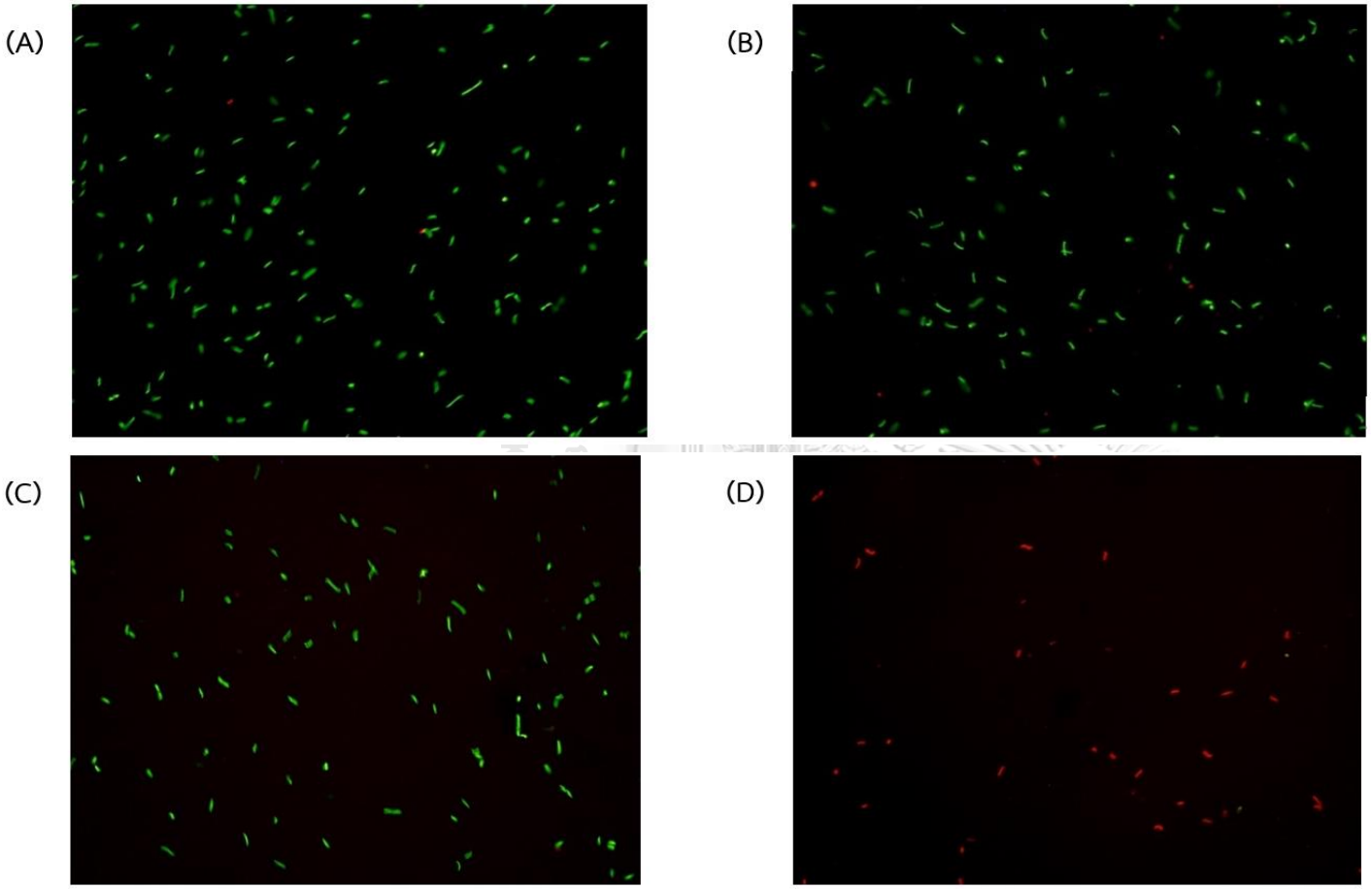


Figure 2.Evaluation of intact leptospires after exposure to stress conditions.

Cell integrity of leptospires under in vitro cultivation (A), temperature shift (B), physiologic osmolarity (C) were determined by Live/Dead fluorescence staining, the green (SYTO9) and red (PI) colors represent intact and dead cells, respectively. Leptospires treated with cool methanol were used as a control of dead or non-intact cells (100% cell death). The experiments were performed in triplicate.

Optimization of centrifugation to minimize cell lysis

Centrifugation was used for initial separation of naturally produced ECVs released in the culture supernatant from *Leptospira* cells. To minimize cell lysis, leptospires were harvested at various centrifugal forces including 3,000×g, 9,000×g and 15,000×g for 15 min as previously described (94). The cell integrity of leptospires in the pellets was determined using Live/Dead BactLight Bacterial Viability Kit. The results indicated that intact cells were decreased as a centrifugation force-dependent manner (Fig. 3D). At 3,000×g, approximately 94% of leptospires remained intact (green) (Fig. 3A). On the other hand, increased centrifugal force at 9,000×g (Fig. 3B) and 15,000×g (Fig. 3C) resulted in decreasing of cell viability to approximately 85% and 63%, respectively. Therefore, centrifugation at 3,000×g was optimal to minimize cell lysis and then used for removing intact cells to obtain the natural ECVs-containing supernatant for the next step of ECV isolation.

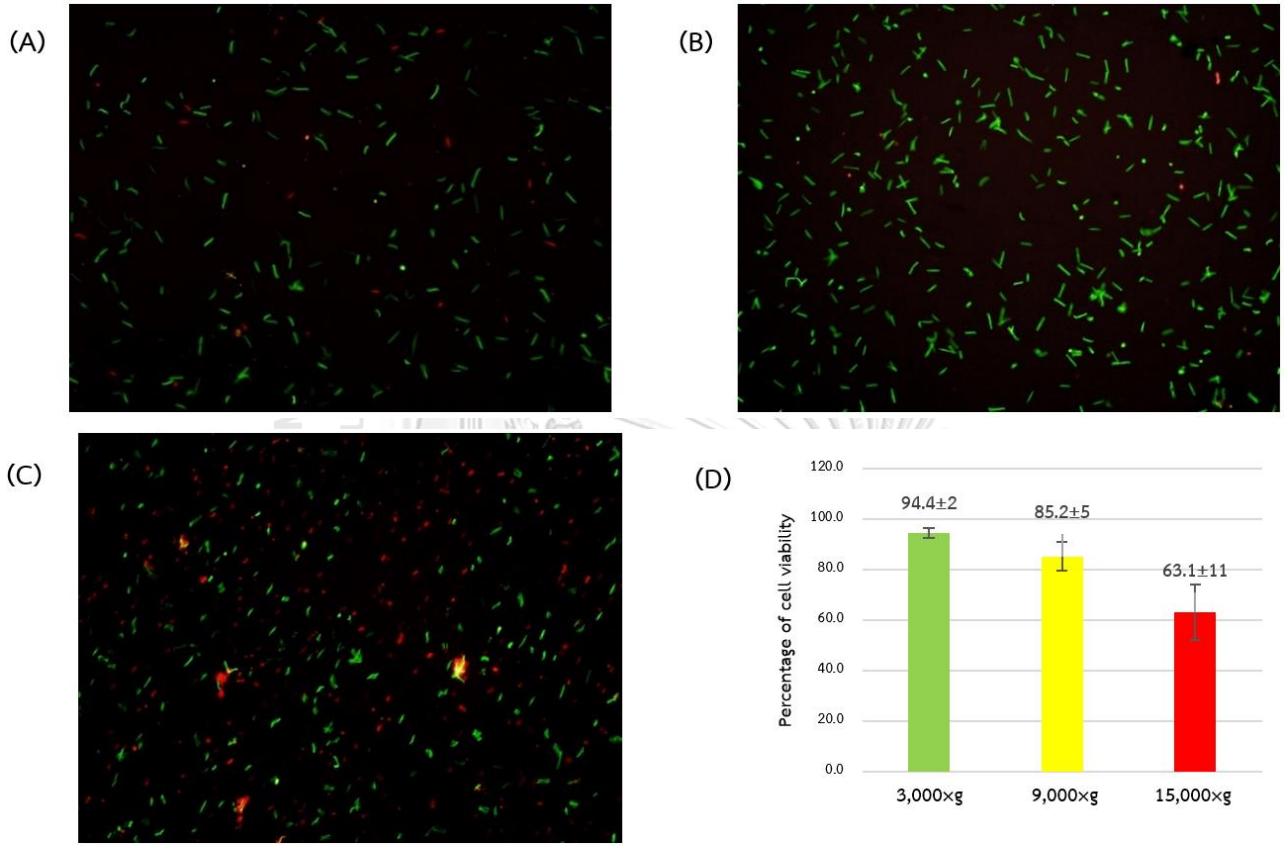


Figure 3 Optimization of centrifugation to minimize cell lysis.

Leptospires in culture media were centrifuged for 15 min at various centrifugal forces; 3,000xg (A), 9,000xg (B), and 15,000xg (C), and stained with Live (SYTO9)/Dead (PI) fluorescence dyes. Cell integrity was assessed by counting intact (green) and dead (red) cells under the fluorescence microscope, and then calculated as the percentage of cell viability (D). performed in triplicate

Isolation and purification of leptospiral extracellular vesicles

After removal of intact cells, the leptospiral ECVs were isolated and purified from the supernatant by filtration through 0.45 μm and 0.22 μm nitrocellulose membranes, ultracentrifugation, and sucrose density gradient centrifugation as described in the flow chart (Fig. 1) in the materials and method. Yellow rings appeared at approximately 35% to 50% sucrose gradient fractions in the samples derived from all three conditions of leptospiral cultivation (Fig. 4, A-C).

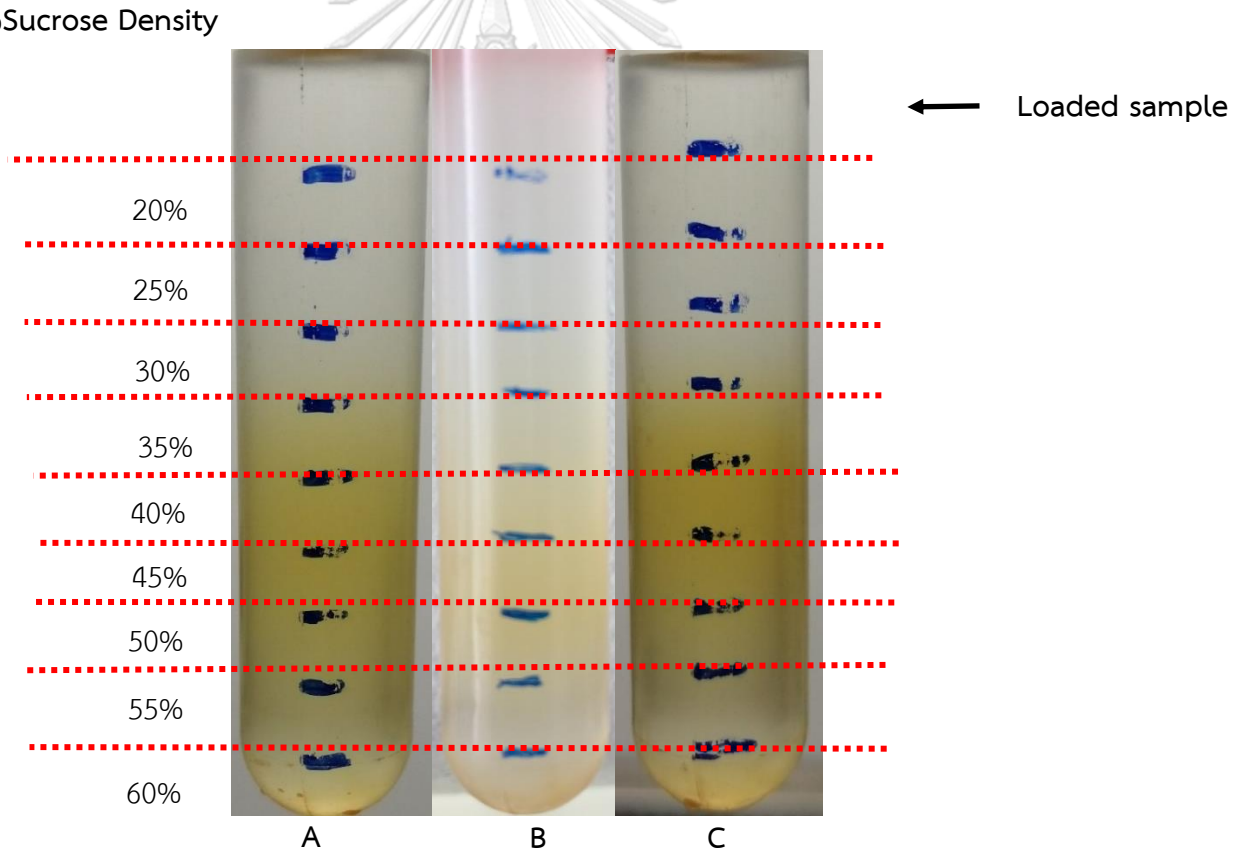
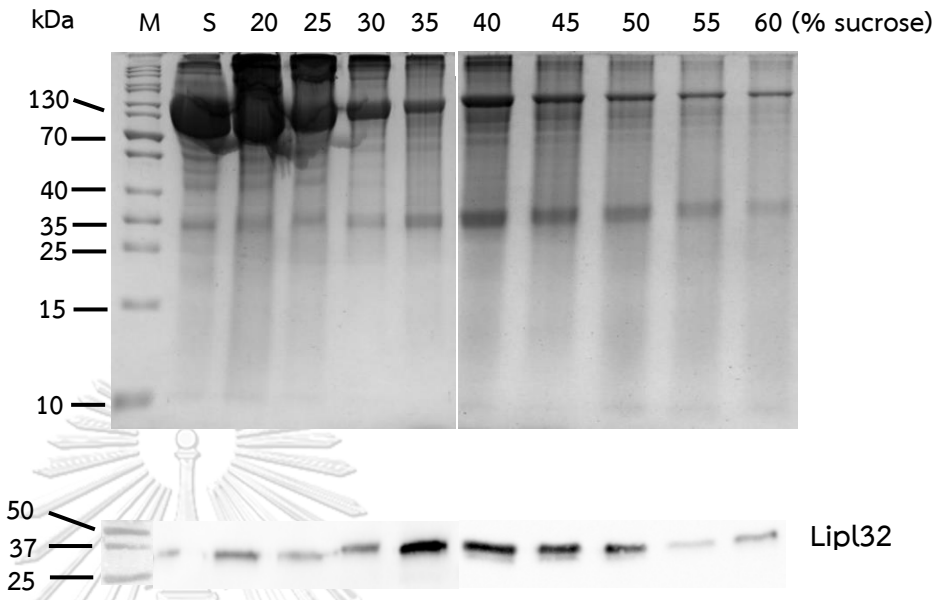


Figure 4. Isolation of leptospiral extracellular vesicles. After filtration and ultracentrifugation, ECVs derived from leptospires cultivation under in vitro growth at 30°C (A), temperature shift to 37°C (B), and physiologic osmolarity (C), were isolated by sucrose density gradient (20-60%) by centrifugation at 77,000×g overnight.

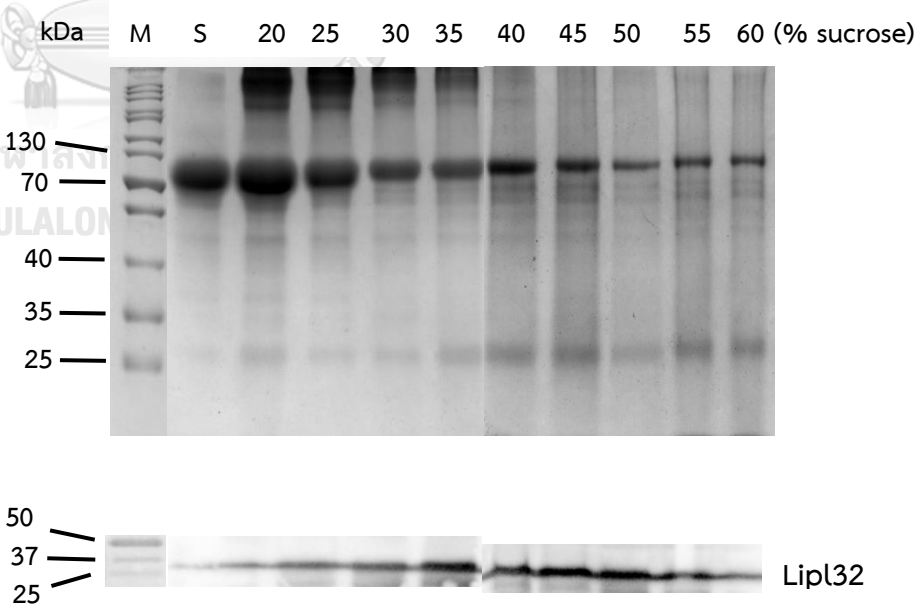
Characterization of purified leptospiral extracellular vesicles

After centrifugation, sucrose gradient fractions were collected by gentle pipetting 800 µl each from top to bottom. Proteins in all fractions separated by SDS-PAGE showed a consistent pattern of leptospiral proteins obtained from all three conditions (Fig. 4A, 4C, 4E). The highest intensity band with approximate size of 130 kDa was observed in the loaded samples before sucrose density gradient centrifugation (lane S), the 20% sucrose fraction, and was gradually decreased in the following fractions of higher sucrose densities (25-60% sucrose). Western blotting against LipL32 (known leptospiral outer membrane lipoprotein) (Fig. 4B, 4D, 4F) showed that LipL32 was present in all fractions with the highest intensity in the 35-45% sucrose fractions. Therefore, these fractions were expected to contain a high number of ECVs and were further characterized by size distribution and transmission electron microscopy.

(A) *In vitro*



(B) Temperature shift



(C) Physiologic osmolarity

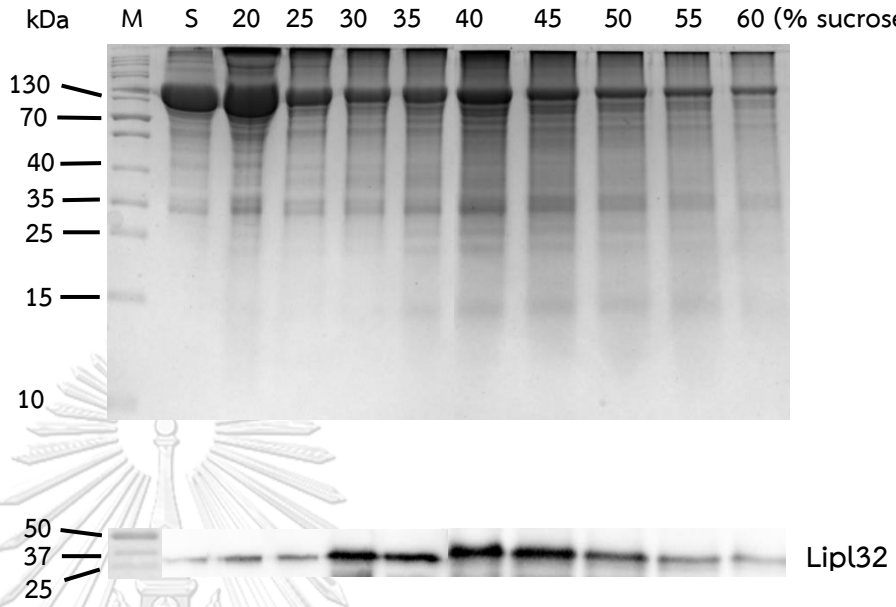


Figure 5. Characterization of purified leptospiral extracellular vesicles.

After sucrose density gradient (20-60%) centrifugation, each fraction (800 µl) was gently collected by pipetting from top to bottom. The fractions obtained from leptospires cultivated *in vitro* (A), temperature shift (C), and physiologic osmolarity (E) were then subjected to 15% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue. The proteins were electrically transferred onto the nitrocellulose membrane and probed with primary monoclonal antibodies against LipL32 followed by HRP-conjugated secondary antibodies. The signal detection was carried out by ECL chemiluminescent system. Lane M, protein marker; lane S, the loaded sample before sucrose density gradient centrifugation; the

following lanes were loaded with proteins obtained from each fraction and labeled according to the percentage of sucrose density.

Size distribution of purified leptospiral extracellular vesicles

To assess size distribution of leptospiral ECVs in 35, 40, and 45% sucrose fractions, the dynamic light scattering and nanoparticle tracking analysis (NTA) was employed. The representative graph showed that the mode sizes of leptospiral ECVs from cultivation at three conditions, *in vitro* growth at 30°C, temperature shift, and physiologic osmolarity, were 86±16, 77±6, and 83±15 nm in diameter, respectively. Moreover, the average concentrations of particles were $3.06\pm1.8\times10^{9}$, $3.74\pm1.6\times10^{9}$, and $2.69\pm0.8\times10^{9}$ particles/ml, respectively (Fig. 6).

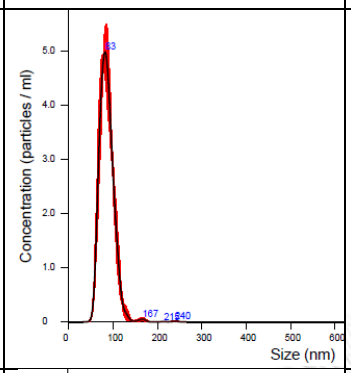
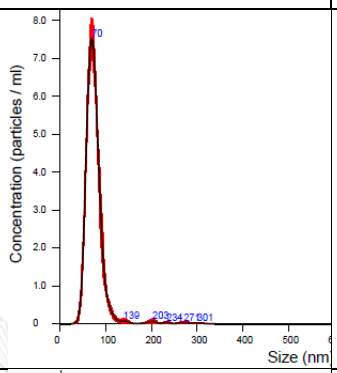
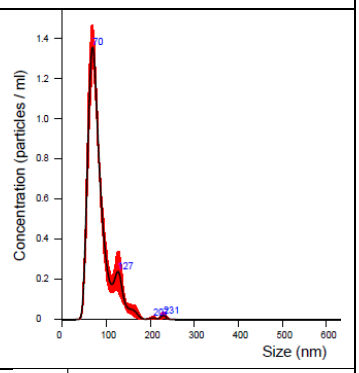
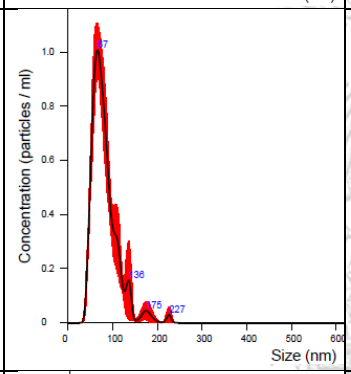
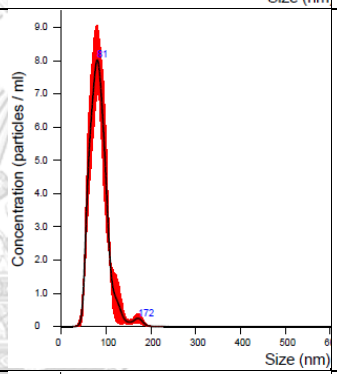
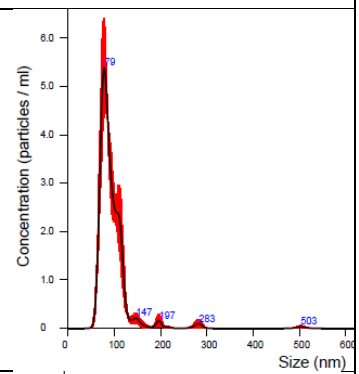
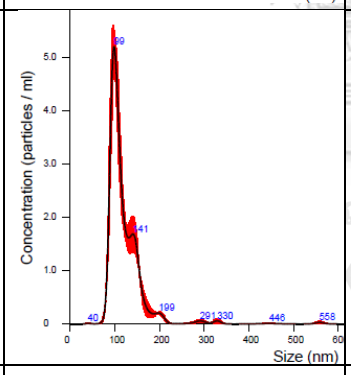
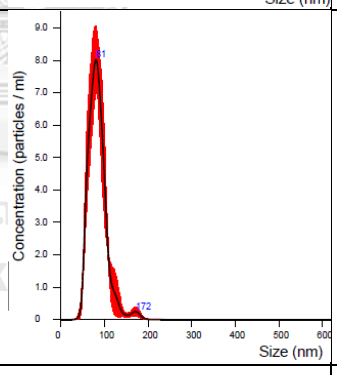
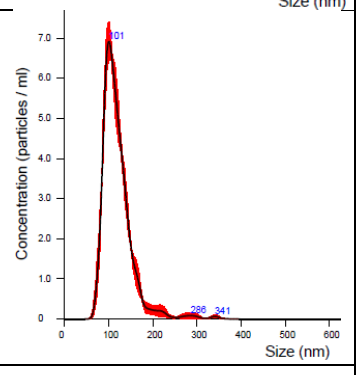
	in vitro 30°C	Temperature shift	Physiologic osmolarity
35% sucrose			
40% sucrose			
45% sucrose			
Mode ± S.D.	83±16 nm	77±6 nm	83±15 nm
Concentration (particles/ml)	$3.06\pm1.8\times10^{9}$	$3.74\pm1.6\times10^{9}$	$2.69\pm0.8\times10^{9}$

Figure 6. Size distribution of purified leptospiral extracellular vesicles measured with dynamic light scattering and nanoparticle tracking analysis (NTA)

The morphological characterization using transmission electron microscopy (TEM) with negative staining revealed that the leptospiral ECVs had spherical shape with averaged size 87.4±29 nm in diameter which corresponded to the size distribution using NTA (Fig. 7A-7E). The intact *Leptospira* with hook ends were shown for comparison (Fig. 7F)

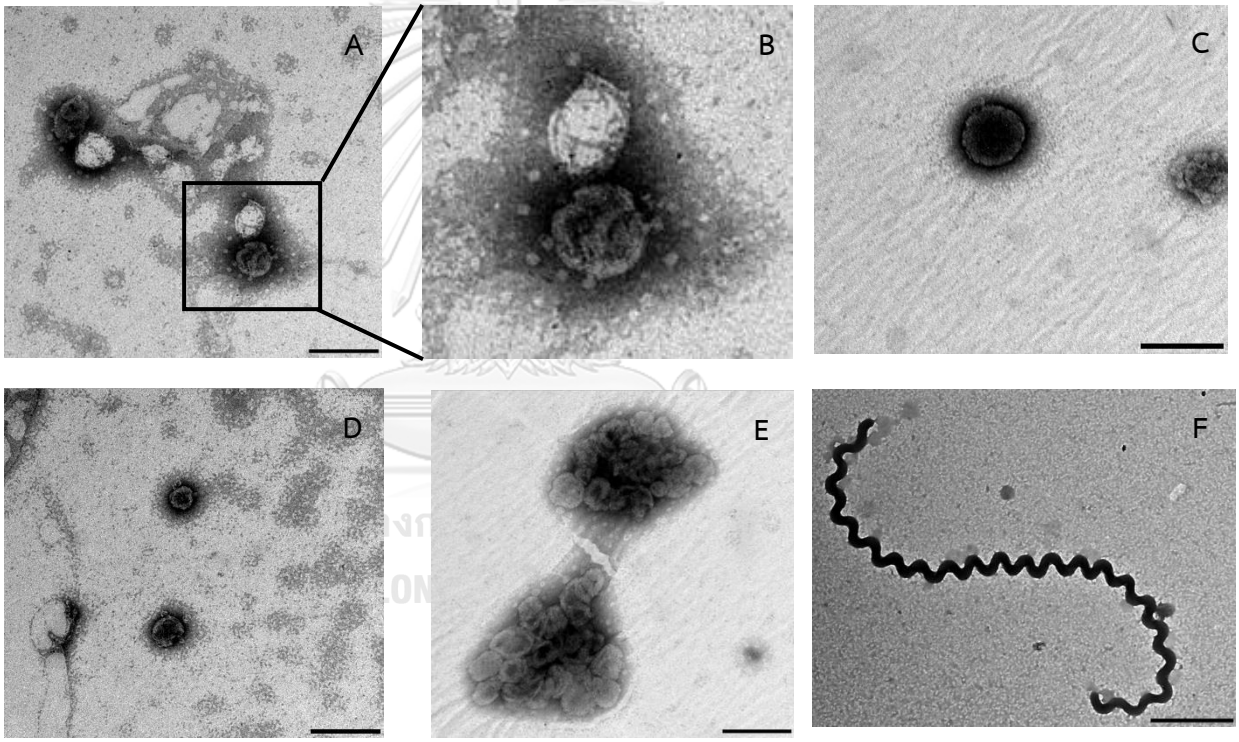


Figure 7. Morphology of purified leptospiral extracellular vesicles. The electron micrograph showed typical spherical shape of leptospiral ECVs (A-E) and intact cells of L. interrogans serovar Pomona (F). The black bars represent 100 nm (A-E) and 2 μm (F) in length.

Identification of proteins in leptospiral ECVs

To identify the proteins in purified leptospiral ECVs, the purified leptospiral ECVs in 35-45% sucrose fractions were enzymatically digested and then determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The raw data were analyzed by MaxQuant and compared to available protein database of *L. interrogans* serovar Copenhageni Fiocruz L1-130 for the identification of leptospiral proteins. The result showed that 690 proteins were harbored in leptospiral ECVs. In addition, a total of 690 proteins were predicted for their subcellular localization using five bioinformatics tools including POSRTb version 3.0.2, CELLO, Gneg-mPloc, SOSUI-Gneg, SignalP. The majority of these proteins were predicted to localize in the cytoplasm (57.9%) followed by the outer membrane (14.8%), inner membrane (14.5%), unknown (5.1%), periplasm (4.0%), and extracellular proteins (3.6%) (Fig.8). The high abundance proteins identified in the purified leptospiral ECVs were DNA-directed RNA polymerase subunit beta, 60 kDa chaperonin, transcription elongation factor GreA, hemolysin, ribosomal protein S1, glutamate synthase (NADPH) alpha chain, translation initiation factor IF-2, uncharacterized protein, and LipL71. Furthermore, in comparison to total proteins identified in *L. interrogans* serovar Pomona whole cells, purified leptospiral ECVs enriched outer membrane proteins (14.8% vs 5.8%), inner membrane proteins (14.5% vs 4.5%), periplasmic proteins (4.0% vs 2.8%), extracellular proteins (3.6% vs 2.6%), but depleted cytoplasmic

proteins (57.9% vs 80.2) and proteins with unknown subcellular localization (5.1% vs 6.7%) (Fig. 9).

To assess biological functions in various bacterial processes, a total of 690 identified proteins were categorized by genome annotation according to KEGG pathway using the UniProt (http://www.uniprot.org/). According to respective percentages of representative functional groups (Fig. 10), the proteins identified in leptospiral ECVs were mostly assigned in undefined category (49.6%) followed by translation (10.6%), carbohydrate metabolism (9.1%), amino acid metabolism (4.6%), metabolism (3.0%), nucleic acid metabolism (2.9%), metabolism of cofactors and vitamins (2.8%), replication and repair (2.6%), cellular processes (2.5%), lipid metabolism (2.3%), transcription (2.3%), energy metabolism (2.0%), folding, sorting and degradation (2.0%), genetics information processes (1.2%), glycan biosynthesis and metabolism (0.7%), metabolism of terpenoids and polyketides (0.6%) metabolism of other amino acid (0.4%), membrane transport (0.4%), and cell motility (0.3%), respectively.

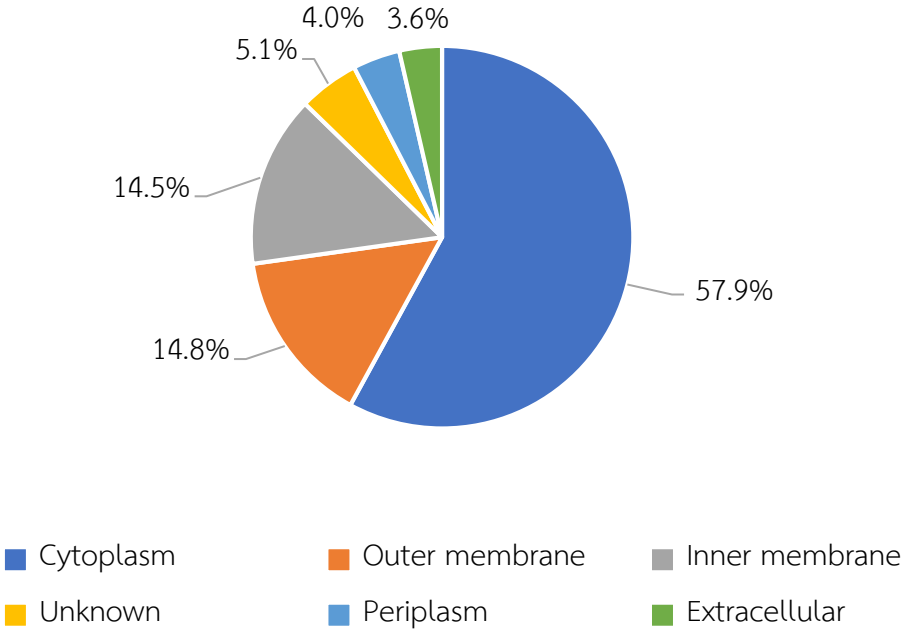


Figure 8. Diagram showing the percentage of predicted subcellular localization of proteins in purified leptospiral ECVs.

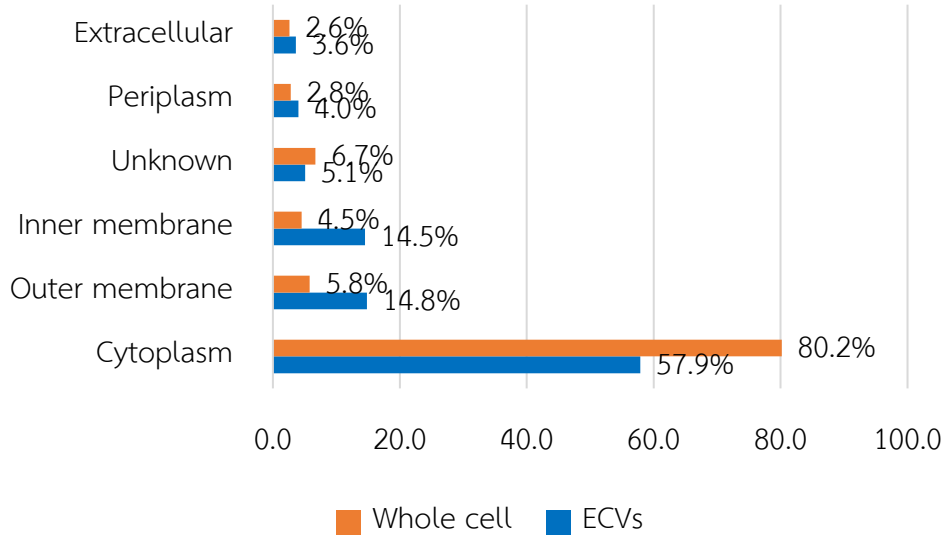


Figure 9. The predicted subcellular localization of proteins in purified leptospiral ECVs compare to those of L. interrogans serovar Pomona whole cells.

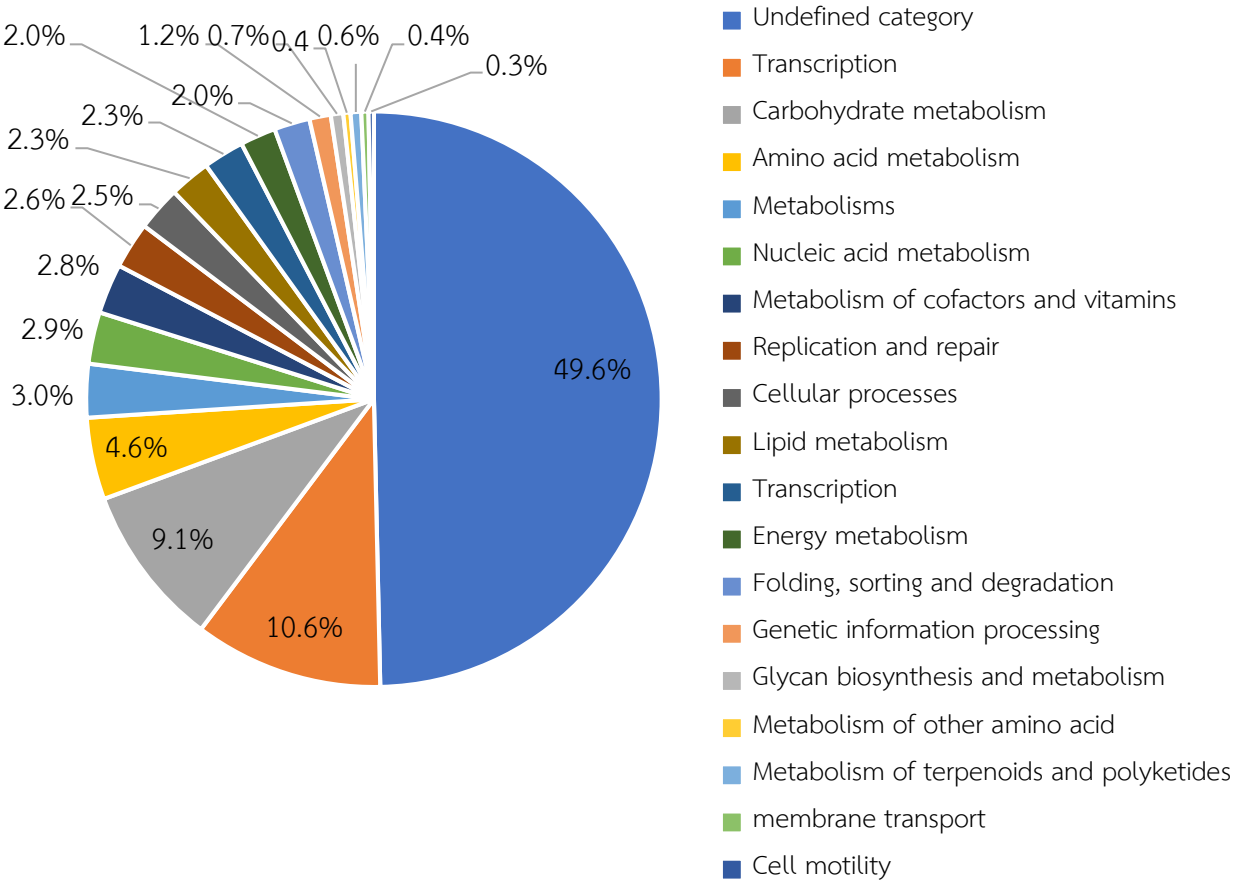


Figure 10. Diagram depicting the percentage of biological functions of proteins in purified leptospiral ECVs based on KEGG pathway annotation.

Relative quantification of proteins in purified leptospiral ECVs under three conditions

To relatively quantify proteins in purified leptospiral ECVs under three conditions, triplex stable isotope dimethyl labeling approach was employed. Purified leptospiral ECVs in 35-45% sucrose fractions from each condition were enzymatically digested and then dimethyl labeled with three different isotopes before identification using LC-MS/MS. The raw data were analyzed by MaxQuant. The average normalized proteins ratio from the three conditions with False Discovery Rate (FDR) < 1% and adjusted *P*-value of less than 0.01 as determined by moderated *t* test were shown in Fig.11-13. The relative quantification of proteins in leptospiral ECVs produced in response to temperature shift compared with those from *in vitro* grown at 30°C showed that a total of 83 proteins were differentially expressed. Of these, 55 proteins were up regulated, and 28 proteins were down regulated in temperature shift condition (Fig. 11). These 22 up-regulated proteins were functionally categorized into undefined category followed by metabolism and genetics information processes, respectively (Table 2). The highly up regulated protein (3.39-fold) was diaminopimelate decarboxylase (lysA).

The relative quantification of proteins in leptospiral ECVs produced in response to physiologic osmolarity compared with those from *in vitro* grown at 30°C revealed that 17 and 89 out of 106 proteins were significantly up- and down-

regulated, respectively (Fig. 12). The uncharacterized protein (LIC_13022) was shown highly up regulated (1.78-fold) followed by transketolase alpha subunit protein (tktA) (1.52-fold). The 17 up-regulated proteins were categorized in carbohydrate metabolism, amino acid metabolism, lipid metabolism, cell motility and unidentified category (Table 3).

In addition, the relative quantification of proteins from leptospiral ECVs under the stress conditions (temperature shift and physiologic osmolarity) were compared. The relative normalized ratio showed that a total of 89 proteins were 3 up regulated and 86 down regulated in physiologic osmolarity (Fig. 13). Of these, The uncharacterized protein (LIC_13022) was shown highly up regulated (1.78-fold) followed by transketolase alpha subunit protein (tktA) (1.52-fold) in the physiologic osmolarity condition, whereas the sulfate ABC transporter periplasmic sulphate-binding protein (LIC_12529) was the most up-regulated (2.9-fold) in the temperature shift condition (Table 4**)**

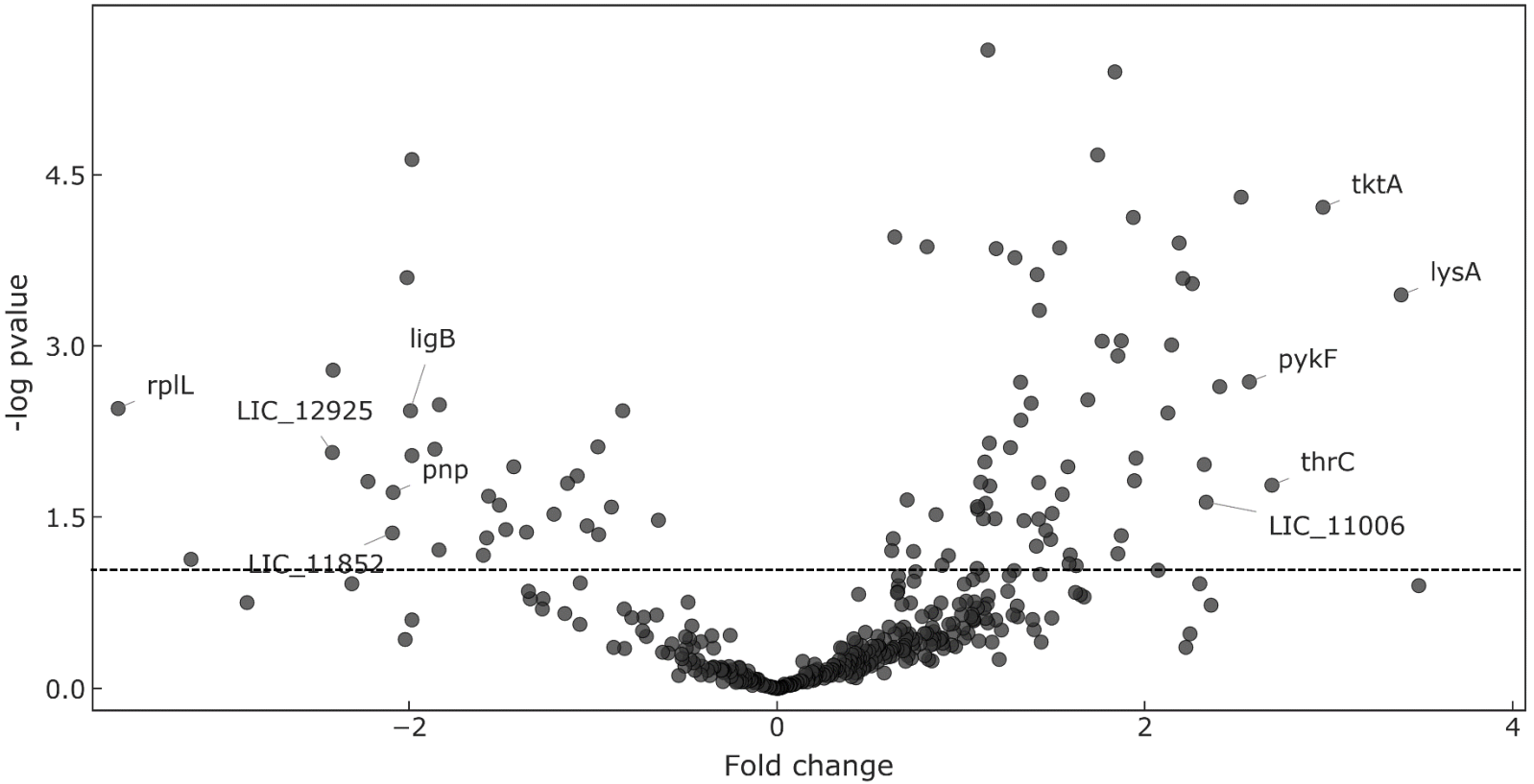


Figure 11. Relative quantification of protein containing purified leptospiral ECVs response to temperature shift normalized with in vitro grown.

Table 2 List of top 10 known proteins differentially expressed in leptospiral ECVs response to temperature shift normalized with in vitro grown

Protein IDs	Gene names	Descriptions	Relative fold change	functions
Q72P58	lysA	tr|Q72P58| Diaminopimelate decarboxylase	3.9	Carbohydrate metabolism
Q72TV3	tktA	tr|Q72TV3| Transketolase alpha subunit protein	3.0	Carbohydrate metabolism
Q72V19	thrC	tr|Q72V19| Threonine synthase	2.69	Amino acid metabolism
Q75FD0	pykF	tr|Q75FD0| Pyruvate kinase	2.56	Carbohydrate metabolism
Q72TL2	LIC_11006	tr|Q72TL2| Transcriptional regulator (FUR family)	2.3	Transcription
Q72R58	LIC_11890	tr|Q72R58| Flagellin	-2.0	Cell motility
Q72V39	ligB	tr|Q72V39| Ig-like repeat domain protein	-2.0	Undefined category
Q72NX	pnp	sp|Q72NX7| Polyribonucleotide nucleotidyltransferase	-2.0	Folding, sorting and degradation
Q72R96	LIC_11852	tr|Q72R96| O-acetylhomoserine (Thiol) lyase	-2.0	Amino acid metabolism
Q72NB1	LIC_12925	tr|Q72NB1| Citrate synthase	-2.4	Carbohydrate metabolism
Q72UA9	rplL	sp|Q72UA9| 50S ribosomal protein L7/L12	-3.6	Translation

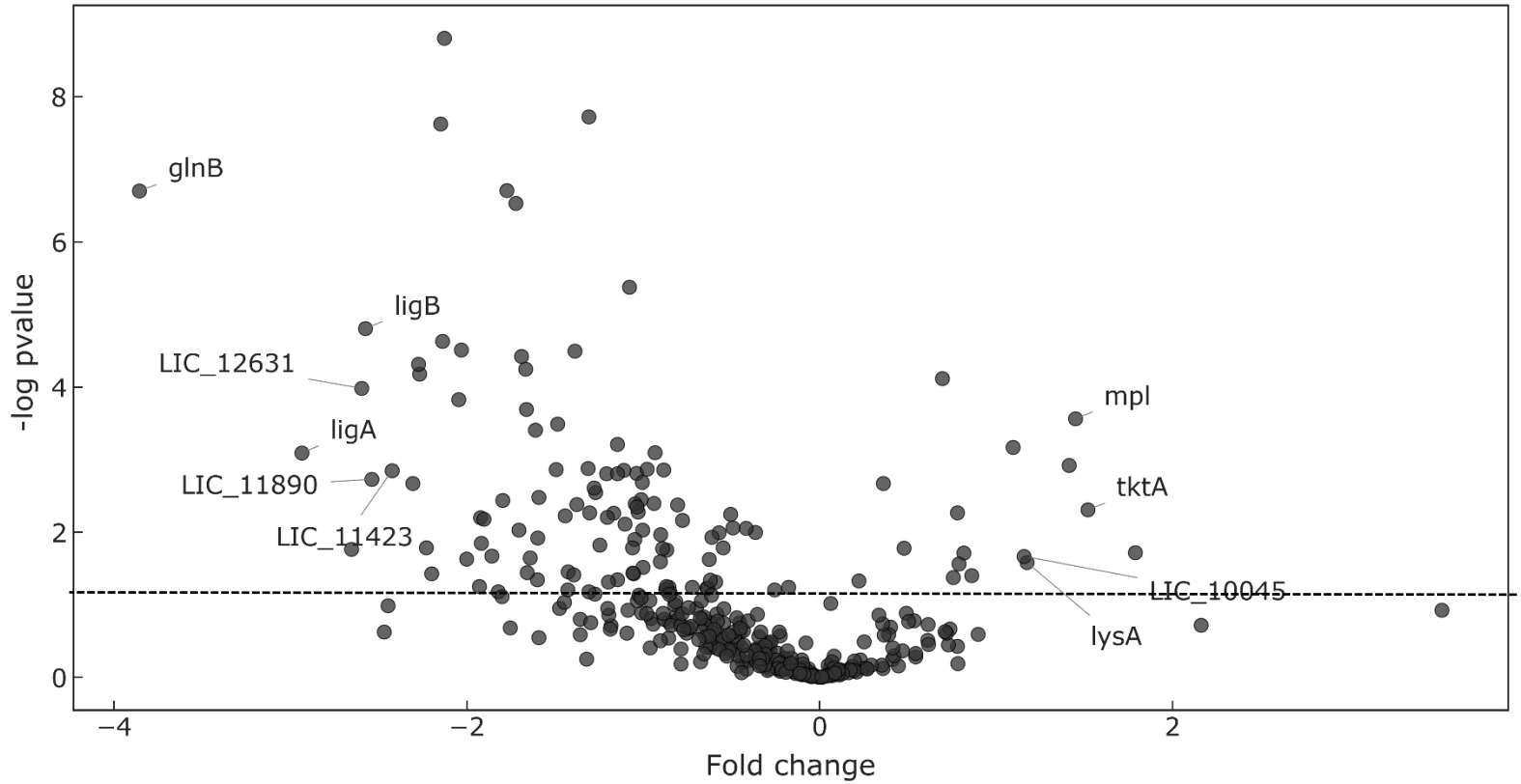


Figure 12. Relative quantification of protein containing purified leptospiral ECVs response to physiologic osmolarity normalized with in vitro grown

Table 3. List of top 10 known proteins differentially expressed in leptospiral ECVs response to physiologic osmolarity normalized by in vitro grown.

Protein IDs	Gene names	Descriptions	Relative fold change	functions
Q72TV3	tktA	tr|Q72TV3|Transketolase alpha subunit protein	1.5	Carbohydrate metabolism
Q72M79	mpl	tr|Q72M79| UDP-n-acetylmuramate:l-alanyl-gamma-d-glutamyl-meso-diaminopimelate ligase	1.4	Metabolism
Q72P58	lysA	tr|Q72P58| Diaminopimelate decarboxylase	1.1	Amino acid metabolism
Q72W92	LIC_10045	tr|Q72W92| dTDP-glucose 4-6-dehydratase	1.1	Amino acid metabolism
Q72SG0	LIC_11423	tr|Q72SG0|Glycine rich RNA-binding protein	-2.4	Unidentified
Q72R58	LIC_11890	tr|Q72R58| Flagellin	-2.5	Cell motility
Q72V39	ligB	tr|Q72V39| Ig-like repeat domain protein	-2.6	Unidentified
Q72P45	LIC_12631	tr|Q72P45| Hemolysin	-2.6	Lipid metabolism
G1UB65	ligA	tr|G1UB65| Ig-like repeat domain protein	-2.9	Unidentified
Q72V62	glnB	tr|Q72V62| Nitrogen regulatory protein pII	-3.9	Unidentified

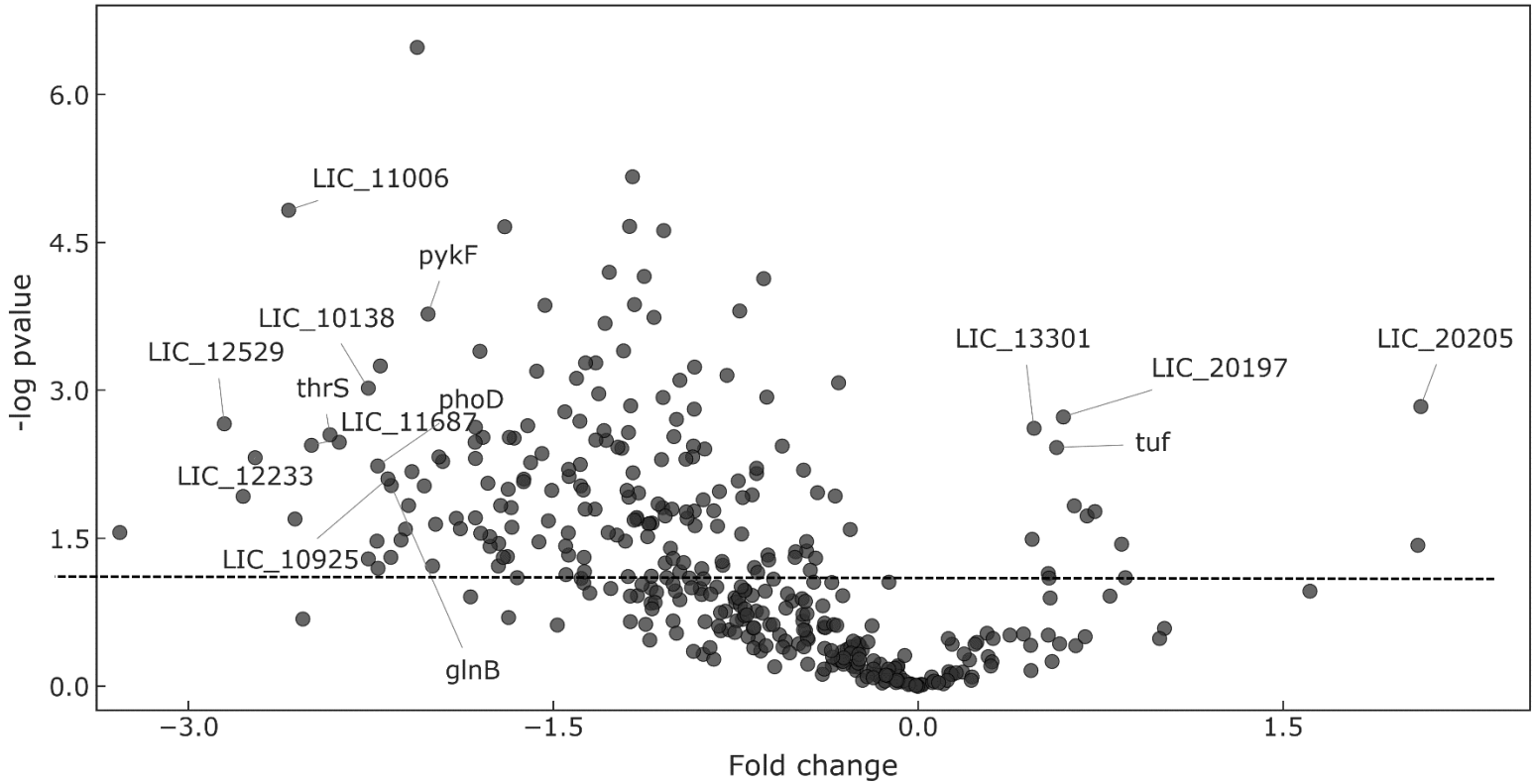


Figure 13. Relative quantification of protein containing purified leptospiral ECVs response to physiologic osmolarity normalized with temperature shift

Table 4. List of top 10 known proteins differentially expressed in leptospiral ECVs response to physiologic osmolarity normalized by temperature shift.

Protein IDs	Gene names	Descriptions	Relative fold change	functions
Q72V62	glnB	tr|Q72V62| Nitrogen regulatory protein pII	-2.16	Unidentified
Q72TU1	LIC_1092	tr|Q72TU1| Metallo-beta-lactamase	-2.1	Unidentified
Q72PD0	trpD	sp|Q72PD0| Anthranilate phosphoribosyltransferase	-2.2	Amino acid metabolism
Q72LZ6	pho	tr|Q72LZ6| Phosphodiesterase	-2.2	Metabolism of cofactors and vitamins
Q72W03	LIC_10138	tr|Q72W03| HD-GYP hydrolase domain protein	-2.2	Unidentified
Q72PK6	thr	sp|Q72PK6| Threonine--tRNA ligase	-2.4	Translation
Q72RQ7	LIC_11687	tr|Q72RQ7| Endonuclease	-2.5	Metabolism
Q72TL2	LIC_11006	tr|Q72TL2| Transcriptional regulator (FUR family)	-2.6	Transcription
Q72Q79	LIC_1223	tr|Q72Q79| Fructose-bisphosphate aldolase	-2.7	Carbohydrate metabolism
Q72PE2	LIC_12529	tr|Q72PE2| Sulfate ABC transporter periplasmic sulphate-binding protein	-2.9	Energy metabolism

CHAPTER VI

DISCUSSION

Bacterial extracellular vesicles (ECVs) are normally released from the membrane compartment of bacteria to extracellular environment and simultaneously contain multiple active molecules (2). Recently, the ECVs are known as one of the bacterial secretion systems (1). Owing to harboring multiple active molecules, ECVs carry virulence factors and play a critical role in pathogenesis of bacterial infections, such as cytotoxicity, adherence to host cells, complement binding, promoting bacterial replication in host cell, vacuolating activity, and nutrient acquisition (10, 11, 70, 97, 98). Moreover, the cargo of ECVs were shown to be LPS, lipoprotein, peptidoglycan, and nucleic acids (DNA and/or RNA) (3-5). These molecules are well recognized as pathogen-associated molecular patterns (PAMPs) that can specifically be engaged with pathogen recognition receptors (PRRs) expressed on the surface of host epithelial cells and immune cells, which consequently induce the innate and adaptive immune responses (79). Bacterial ECVs have been exploited as a vaccine platform to combat the pathogens (12, 77, 85). Hence, characterization of the cargo components in ECVs is useful for insight into the

pathogenic mechanism and development of diagnostic tests and vaccines for leptospirosis.

Previously, leptospiral outer membrane proteins (OMPs) were studied using chemically induced leptospiral ECVs from *Leptospira kirchneri* as a model (20). Moreover, the chemically induced leptospiral ECVs were used as acellular vaccines in a hamster model (21). However, naturally produced leptospiral ECVs have not been characterized.

To study ECVs, the isolation and purification of ECVs are crucial steps. However, the standard methods for isolation and purification of ECVs are still lacking (99). Currently, the potential strategy to isolate and purify ECVs with high purity is a combination of several methods including filtration, ultracentrifugation, density gradient centrifugation, and size exclusion chromatography (100, 101). In this study, we first used low speed centrifugation followed by vacuum filtration to remove intact cells. ECVs were then isolated from the filtered supernatant by ultracentrifugation, which was a limited step because contaminated components including flagella, secreted proteins, and protein aggregates were co-purified with the ECVs (7, 73). Therefore, additional step of purification is required. The density gradient centrifugation was most often employed to purify ECVs in previous studies (73, 102-104). This purification method relied on sedimentation of macromolecules

based on buoyant density of macromolecule (7, 100). Previously, leptospiral ECVs produced by treating *L. kirchneri* with alkaline plasmolysis buffer pH 9.0 and isolated by sucrose gradient ultracentrifugation were mostly sedimented onto 1.19 g/ml of sucrose (approximately 35% sucrose) (20). In this study, sucrose density gradient resulted in sedimentation and enrichment of leptospiral ECVs at 35%-50% sucrose (1.15-1.48 g/ml of sucrose) (Fig. 4) indicated by high intensity of LipL32 on the Western blot (Fig. 5). The purified leptospiral ECVs in these sucrose fractions were further confirmed by morphological characterization using negative staining and TEM revealing typical spherical shape with nano-size (about 100 nm) in diameter (Fig. 7) similar to ECVs of other bacteria **(72, 105)**. Their size distribution based on dynamic light scattering also demonstrated homogeneity of leptospiral ECVs with approximately 100 nm (Fig. 6).

Bacteria normally secrete ECVs into extracellular environment in response to harmful signals (106). Several stress conditions, such as temperature shift, oxidative stress, antibiotics treatment, were simulated to induce ECVs (100) (8, 9). Temperature shift was performed as a common stress condition that leptospires generally encounter during infection (93). Temperature shift of cultivation from 30°C (*in vitro* growth at 30°C) to 37°C (temperature shift) resembles environment changing of leptospires moving from external environment into the host. Physiologic or isotonic

osmolarity (EMJH medium supplemented with 120 mM NaCl) mimicked the hostile host environment that leptospires expose in the mammalian host (92, 98). Physiologic osmolarity was previously shown to up-regulate the expression of Lig proteins, which play a critical role in pathogenesis of leptospirosis including binding to host extracellular matrix (ECM) components, such as plasminogen, fibrinogen, vitronectin, laminin, and collagens (type I, III, IV) (49, 50, 92, 107). In this study, proteins of leptospiral ECVs produced under the temperature shift and physiologic osmolarity were compared to those produced under *in vitro* growth at 30°C by relative quantification using dimethylation labelling and determined using LC-MS/MS.

In previous studies, leptospires were treated with chemical reagents that caused cell lysis and subsequently reformed to ECVs so called chemically induced ECVs (21, 85) . Due to these ECVs derived from cell lysis, they were non-natural and contained a large number of cytoplasmic proteins. In this study, cell lysis at each experimental step of exposure to stress conditions and isolation of leptospiral ECVs was minimized and cell integrity was determined by viability fluorescence staining using SYTO9 and propidium iodide (PI) to ensure that ECVs were isolated from intact leptospires. Despite gentle manipulation, cell lysis is unavoidable. Therefore, each technique was performed in triplicate to improve the reliability of our results. The viability fluorescent staining showed that leptospires mostly remained intact after

exposure to stress conditions (approximately 90% and 95% under temperature shift and physiologic osmolarity, respectively) (Fig. 2) and after removing the intact cells using centrifugation at 3,000×g for 15 min (94%) (Fig. 3A).

In this study, we aimed to identify protein harbored in leptospiral ECVs. Recently, gel-based proteomic analysis was used to characterize proteins in chemically induced leptospiral ECVs (20, 21). However, this approach has several limitations because it is laborious, time consuming, and less compatible with hydrophobic membrane proteins leading to poor reproducibility and biased protein abundance (108, 109). Therefore, in-solution based proteomic analysis approach was employed to identify proteins in the leptospiral ECVs in this study. Our finding revealed 690 proteins in the purified leptospiral ECVs. After further predicted subcellular localization in comparison to *L. interrogans* serovar Pomona whole cells, the leptospiral ECVs enriched outer membrane proteins (14.8% vs 5.8%) and inner membrane proteins (14.5% vs 4.5%) (Fig. 9), whereas the citrate buffer induced leptospiral ECVs contained 2.17% of predicted outer membrane proteins (21). These findings may suggest the biogenesis of leptospiral ECVs. Although the outer membrane proteins were not highly enriched as in ECVs of other bacteria **(110, 111)**, the enrichments of outer membrane proteins coupled with inner membrane proteins

might suggest that leptospiral ECVs were originated from both outer and inner membranes. However, further investigations are required to confirm this speculation

To relatively quantify the protein abundance of leptospiral ECVs in response to three conditions, dimethylation labelling followed by LC-MS/MS was employed. Quantitation of relative protein abundance of leptospiral ECVs produced under *in vitro* growth at 30°C and stress conditions revealed differential expression of proteins (Fig. 11, 12, 13). Of these, proteins associated with metabolism functions, especially diaminopimelate decarboxylase (3.9-fold change), were significantly upregulated in leptospiral ECVs produced under temperature shift compared with those under *in vitro* growth at 30°C (Table 2). The diaminopimelate decarboxylase has been reported to play a role in bacterial survival by catalyzing the decarboxylation of D,L-diaminopimelate (D,L-DAP) to form L-lysine, which are important precursors of peptidoglycan, housekeeping proteins, and virulence factors (112-114). In response to physiologic osmolarity, transketolase alpha subunit was the highest up-regulated protein with 1.5-fold change compared to *in vitro* growth at 30°C (Table 3). This protein is an enzyme that catalyzes several key reactions of nonoxidative branches of the pentose phosphate pathway. Previous reports on transketolase indicated that this enzyme was associated with RpoS gene in *Salmonella enterica* serovar Typhimurium and *E coli* to promote bacterial survival in stress conditions (115, 116)

suggesting that the transketolase was required for virulence. The diaminopimelate decarboxylase was also significantly up-regulated (1.1-fold change) in response to physiologic osmolarity. In relative comparisons between physiologic osmolarity and temperature shift, the most significant up-regulated protein after temperature shift was sulfate ABC transporter periplasmic sulphate-binding protein (2.9-fold). Moreover, nitrogen regulatory protein pII was 2.16-fold up-regulated. This finding was consistent with 3.7-fold up-regulation of nitrogen regulatory protein pII of *L. interrogans* serovar Lai cultivated under temperature shift condition (93).

Furthermore, although the relative quantification was not statistically significant, several virulence factors were identified in leptospiral ECVs including Lig proteins, LipL21, LipL32, Lip41, and hemolysin. Lig proteins (LigA and LigB) were found to be lipoproteins consisting 12-13 Ig-like domains with adhesion activity to host ECMs including plasminogen, fibronectin, laminin, and collagen and involved in bacterial colonization (49, 50, 107). In addition, Lig proteins were investigated as vaccine candidates, which provided 60-100% survival protection (94). LipL21 was previously reported to have inhibitory activity against myeloperoxidase of neutrophils (59). LipL32 acted as adhesins by binding to ECMs and induced inflammatory responses via toll like receptor 2 (51, 117, 118). LipL41 showed binding affinity to hemin suggesting a possible role in iron regulation and storage (119). Leptospiral

hemolysin revealed pore-forming activity on erythrocyte and induced pro-inflammatory cytokines via toll like receptor 2 and 4 (120, 121). Several hypothetical proteins found in leptospiral ECVs might be putative virulence factors and associated with pathogenesis of leptospirosis.

In summary, the present study showed successful isolation and purification of leptospiral ECVs with enrichment of the outer and inner membrane proteins. The leptospiral ECVs produced under *in vitro* growth at 30°C and in response to stress conditions, temperature shift to 37°C and physiologic osmolarity harbored several proteins which may play a role in pathogenesis of leptospirosis. This knowledge will be useful for insight into pathogenesis of the disease and vaccine development against leptospirosis in the future.

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