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Article

Extracellular Secretomes of *Leptospira Interrogans* Serovar Icterohemorrhagiae Respond to the *In Vivo* Mimic of Physiological Osmolarity and Temperature Transition

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Abstract: Leptospirosis remains an important worldwide zoonotic disease caused by *Leptospira* spp affecting human and animals. This research aims to study the virulent-associated secreted proteins (protein secretome) of pathogenic *Leptospira interrogans* serovar Icterohemorrhagiae strain RGA (*Leptospira* RGA) transition from the environment to mammalian physiological osmolarity, temperature (37 °C) and carbon dioxide concentration (5% CO_2) conditions for 24 h. Mass Spectrometry and bioinformatics approaches, we identified 69 potential secreted proteins from the culture supernatant of the *Leptospira* RGA isolate. We discovered transporters and porins such as phosphate porin, outer membrane efflux, ompA family protein, and polymer-forming cytoskeletal family protein under hyperosmotic condition. Under heat stress, degradation enzymes included zinc metallopeptidase, M23 family (LA3456, LA0709), Rhs family protein (LA1765), thermolysin metallopeptidase; α/β hydrolase family (LA1345, LA2501). Oxidative stress response proteins induced by osmolarity and temperature shifts included chaperon GrpE, DnaK (LA3705), antioxidants, i.e., thiol-specific redoxin, and peroxiredoxin (LA2809). In response to the *in vivo* transition, metabolic and other enzymes involved in energy production (COG:C), amino acid metabolism and transport (COG:E), and lipid metabolism and transport (COG:I), as well as moonlighting proteins functionally binding to plasminogen and fibronectin and regulating transcription, were also discovered. An overview of secreted proteins will supplement our understanding of *Leptospira* biology and pathogenesis during infection and also in response to environmental stimuli and their potential virulent determinants have the potential for developing leptospirosis vaccines and diagnosis.

Keywords: *Leptospira* spp., secretome; virulent-associated secreted proteins; *in-vivo* mimic mammalian condition

1. Introduction

Leptospirosis remains one of the neglected zoonotic disease worldwide, endemic in subtropical or tropical countries particularly in the Americas and Asia with epidemic potential. There is an estimated 500,000 cases of which mortality is greater than 10% annually

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[1,2]. Thailand is an endemic leptospirosis among Southeast Asian Countries [3]. Thai Bureau of Epidemiology has reported 1,603 human leptospirosis cases in 2020 and incidence may represent up to 20% of febrile illness of unknown (undiagnosed/misdiagnosed) origin. The disease is associated with agriculture occupational exposure, seasonal with peak incidence in the late rainy to early winter, occasionally outbreaks following flooding such as in Thailand in August 2006 [4]. In Europe, recreational activities, particularly water sports and travel, are major risk factors for leptospirosis [5].

Leptospirosis is a bacterial infectious disease caused by spirochetes of the genus *Leptospira*. To date, the genus *Leptospira* have been divided into at least 23 species in three groups according to clinical severity: pathogenic that cause the most severe cases, intermediate that cause milder symptoms, and free-living saprophytes species. There are over 300 different serovars currently identified and clustered into 25 serogroups [1,6]. *Leptospira interrogans, L. borgpetersenii* and *L. kirschneri* are pathogenic species of leptospirosis in humans and animals worldwide [7]. Human leptospirosis has a broad range of clinical manifestations, ranging from mild febrile illness to life-threatening such as Weil's disease and Severe pulmonary haemorrhage syndrome [8]. The major influencing complications of leptospirosis include the infecting *Leptospira* serovars, susceptibility of the host, environment and the behavior of the host. *Leptospira* serovar Icterohemorrhagiae is one of the predominant local pathogenic strains in humans and mammals and the infections were associated with the infestation of rodents carrier. Furthermore, serovar Icterohemorrhagiae was linked to severe outcomes such as pulmonary hemorrhage, hepatic jaundice [1].

Leptospirosis is transmitted globally through a wide range of mammalian hosts, including dogs, pigs, cattle and wildlife animal and humans [9]. Maintenance hosts are carrier animals that harbor leptospires in their proximal renal tubules, commonly resulting in shedding of bacteria in the urine and subsequently the environments. Transmission involves either direct or indirect exposure to pathogen that shed in the urine or in the infected animals. *L. interrogans* is known to survive for a period of time in contaminated soil and water [1]. Infection of mammalian host involves an initial shift from ambient low osmolarity and temperature environment to physiological osmolarity at 37 °C and later to as high as 39 °C in hosts during acute febrile illness of acute leptospirosis [8]. Therefore, both osmolarity and temperature are likely to be a key signal to leptospires of transition changes from environments to infective stage. Transcriptomes of *L. interrogans* genes expressions respond to temperature upshift in early stage of infection have been found to be genes encoding proteins that facilitates invasion and establish of disease [10] while microarray study of *L. interrogans* under physiological osmolarity transition demonstrated induction of signal transduction that regulates virulence and also coregulated with temperature induced genes encoding lipoproteins [11].

Extracellular secretome has been found to play an important role in the regulation of many processes during pathogenesis or transition from environment to mammalian host including nutrient uptake, host-pathogen interactions, induction of immune response, immune evasion, and host colonization [12-16]. The secreted proteins provide potential targets for diagnosis, and vaccine development. However, there is a limited knowledge regarding protein expression and secretion in response to physiological condition when the pathogenic *Leptospira* transition from free-living in environmental to invasive stage under mammalian infection.

To identify novel leptospiral virulence determinants from secretome, integrative approaches have been extensive studied such as genome-wide subcellular localization of putative outer membrane and extracellular proteins in *Leptospira interrogans* serovar Lai [17,18]. Transcriptomic and proteomic profiles of *Leptospira* during infections and upon various environmental changes has been also studied in *in vitro* growth models to simulate those encountered within the mammalian host, such as physiological temperature and/or osmolarity upshifts, iron starvation, and the presence of serum components

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[10,11,19-21]. This study, *in vivo* mimic secretome of *Leptospira* RGA was assessed in dialysis solution consisted of bicarbonate-acetate buffer, and 0.1% glucose. This model assumes that the leptospires would be exposed and react similar to what leptospires would encounter within proximal convoluted tubules, where the composition of the glomerular ultrafiltrate most resembles that of dialysis solution during chronic infection. Dataset of potentially secreted proteins of *Leptospira interrogans* in *in vivo* physiological condition and their potential virulent-associated proteins will supplement previously studies of extracellular secretome of *Leptospira* spp. to understand biology and pathogenesis during infection and also in response to environment changes.

2. Materials and Methods

2.1. Antisera

Animal ethic was approved by ethical committee, Faculty of Tropical Medicine, Mahidol University (MUTM2016-100-01). Biosafety was approved by Institute Biosafety Committee of Mahidol University (MU2017-001). Mouse antisera against whole-*Leptospira* and recombinant LipL32 were generated in ICR mice as follows. Whole-cell lysate of *L. interrogans* was prepared as previously described [22]. Recombinant LipL32 protein was produced from transformed *E. coli* carrying *lipl32*-pET23a(+) plasmid as previously described [23]. ICR mice were intraperitoneally immunized with individual alum-adjuvanted immunogens including whole-cell protein and rLipL32, and then boosted for 2 times. Blood was collected at day 28 of immunization schedule. IgG titers were 1:40,000 and 1:320,000 against whole-*Leptospira* antigen and rLipL32, respectively and antigenic specificity was verified by immunoblotting to homologous antigens.

2.2. Dialysis Solution

Dialysis solution consists of 0.1% glucose in a balanced electrolyte, bicarbonate-acetate solution (138 meq/L Na^{2+}, 3 meq/L K^{+}, 3.5 meq/L Ca^{2+}, 1 meq/L Mg^{2+}, 109.5 meq/L Cl^{-}, 35 meq/L HCO^{3-}, 3 meq/L Acetate), closely resemble to protein-free plasma. Dialysis solution was obtained from hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University. Colloidal osmotic pressure (COP) was measured by a membrane colloid osmometer (Osmomat050, Gonotec, Germany). Viscosity was measured at varied shear rate using a cone-plate viscometer (Brookfield Engineering Laboratories, USA).

2.3. Leptospira Cultivation

Pathogenic *L. interrogans* serogroup Icterohaemorrhagia serovar Icterohaemorrhagia str. RGA (Leptospira RGA) was re-isolated on LVW agar [24] and the culture was kept as DMSO stocks (10%) at -70 °C. *Leptospira* serovar was verified by MLVA typing as previously described [25].

Leptospira was cultured in EMJH medium (10% polysorbate-80-albumin enrichment) (Becton, Dickinson and Company, MD, USA) at 30 °C under aerobic condition as previously described [16] and sub-cultured in fresh EMJH media not more than 4 times. Viability and motility of spirochetes were examined under dark-field microscopy. Growth of *Leptospira* cultures was monitored by optical density of 420 nm (OD_{420nm}). The procedures were performed on a laminar flow hood, performing aseptic techniques to minimize risk of other microbial contamination.

2.4. Secretome preparationl

Secretome of *Leptospira* in *in vivo* mimic mammalian host condition was prepared in a dialysis solution. Briefly, late-exponential phase of *Leptospira* cultured in EMJH media was harvested, and washed three times with sterile phosphate buffered saline (PBS) and then the cells pellet was gently resuspended with dialysis fluid to make *Leptospira* suspension at ~1×10^{8} cells/. The *Leptospira* suspension was incubated for 24 h at three different conditions including aerobically at 30°C (to represent osmolarity shift), 37 °C (to represent

osmolarity and temperature shifts), and incubated at 37 °C with 5% CO_2 (to represent *in vivo*-like condition). Cell-free supernatant was collected by low-speed centrifugation at 3,000 ×*g*, 4 °C for 30 min, followed by re-centrifuging the solution at 10,000 ×*g*, 4 °C for 30 min to remove leptospires.

Dialysis solution was concentrated by using a 3 kDa cut-off Amicon Ultra filter at 4 °C (Merck Millipore, MA, USA) and kept as aliquots at -70 °C. Three replicates were performed. Outer membrane proteins contamination in the secretome was assessed by determining LipL32 using anti-LipL32 serum in Western blotting.

2.5. SDS-PAGE

Secretome samples were mixed with SDS loading dye with β-mercaptoethanol, boiled for 5 min and separated on 13% SDS-PAGE (7 × 10 cm) under denaturing condition in Mini-PROTEAN^R Electrophoresis System (Bio-Rad, Hercules, USA) at 10 mAmp/gel for 1.5 h, followed by either Coomassie Brilliant Blue (CBB) R-250 staining. Protein patterns on SDS-PAGE gels were scanned using the GE Healthcare Typhoon Scanner (GE Healthcare, Dornstadt, Germany).

2.9. Western Blotting

The separated proteins on SDS-PAGE gel was electro-transblotted onto nitrocellulose membrane (PALL Corporation, PA, USA). The NC was blocked with blocking reagent (5% skim milk in PBS) at 25 °C for 1 h. The membrane was then incubated with appropriate dilutions of primary antibody (1:2,000 dilutions of anti-LipL32 serum or anti-*Leptospira* serum), followed by 1:2,000 dilution of HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody (Thermo Fisher Scientific, Waltham, MA) and developed signal using chromogenic DAB substrate (Thermo Fisher Scientific, Waltham, MA).

2.10. Tryptic Peptide Preparation

To identify secreted protein, secretome separated in 13% SDS-PAGE gel was excised into 11 pieces, and each gel piece was de-stained in 50% acetonitrile/ 25 mM NH_4HCO_3 until colorless. Freshly prepared 10 mM dithiothreitol in 25 mM NH_4HCO_3 was added to each sample to reduce protein for 30 min at 55 °C, followed by adding freshly prepared 10 mM iodoacetamide in 25 mM NH_4HCO_3 to alkylate the protein in the dark at room temperature for 30 min. The solution was removed and acetonitrile was then added to dehydrate gel pieces until the gel was completely dried. Trypsin solution (0.01 mg/mL) was added to digest the gel pieces at 37 °C for 16 h. Tryptic peptides were extracted by 0.1% formic acid, followed by concentrated and desalting prior to mass spectrometry analysis.

2.11. Mass Spectrometry Analysis

Tryptic digested samples were analyzed by LC–MS/MS system consists of a liquid chromatography part (Dionex Ultimate 3000, RSLCnano System) in combination with an captivespray ionization / mass spectrometer (Model Q-ToF Compact II, Bruker, Germany) at the Proteomics Services, Faculty of Medical Technology, Mahidol University (Salaya Campus), Thailand. The LC separation was performed on a reversed phase column (Acclaim PepMap RSLC 75 µM ×15 cM, nanoViper C18), protected by a guard column (C18 PepMap100, 300 µM × 5 mM, 5 µM). The system was operated at flow-rate of eluted at a flow rate of 0.3 µL min^{-1} and over 50 min gradient was used to separate the peptides.

2.12. Mass Spectrometry Data Analysis

The peptide sequence was searched on in-housed mascot server search engine to identify proteins from the MS/MS data against *L. interrogans* database with 522,795 sequences and 149,535,808 residues using trypsin with maximum missed cleavages allowed at 1. The peptide mass and fragment mass tolerance were set to ± 1.2 Da and ± 0.6 Da. The

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protein identified with at least two different specific peptides and scored greater than 20 was validated. To compare secreted protein quantities in the secretomes (30 °C *vs* 37 °C *vs* 37 °C with 5% CO_2), exponentially modified protein abundance index (emPAI) value for estimation of absolute protein amount in the proteomics was calculated and compared to assess relative abundance [26].

2.13. Computational Predictions

Secreted protein potentially localized extracellularly was verified by using SignalP 4.0 server [27]. Non-classically secreted protein was verified by SecretomeP 2.0 server [28]. Protein sequence contained N-terminus signal peptide sequence was verified as classical secreted protein and the protein with a neural network score exceeding 0.6 cut-off value was verified as non-classical secreted protein. Hypothetical protein was searched for putative ortholog present in *L. interrogans* serovar Lai str. 56601 based on a BlastP alignment threshold and minimum amino acid sequence homology over 80%. Protein was allocated into clusters of orthologous groups (COG) functional category using MicroScope platform [29].

3. Results

3.1. Physiochemical Properties of Dialysis Solution

The dialysis solution is clear and had a viscosity at a shear rate of 150 s^{-1} of 0.97 cP compared to 1.02 cP of 0.9% saline solution (NSS) (Table 1). The pH was physiological with a slightly alkaline range of 7.7–7.8 compared with EMJH media (7.5) and plasma (7.4) (Table S1). Dialysate fluid and in the presence of 1% serum had colloidal osmotic pressure (COP) values of 0.23 and 0.28 mmHg, respectively, similar to the COP of 0.25 mmHg in NSS, whereas EMJH containing 10% BSA had a COP of 2.4 mmHg. The total oncotic pressure of an average capillary is about 28 mmHg with albumin (55%) contributing approximately 22 mmHg of this oncotic pressure.

Table 1. Physiochemical properties of dialysis solution compared to EMJH media at 25 °C.

Media/ Dialysis solution (DL)	pH	Viscosity at shear rate of 150 s^{-1} (cP)	Colloid osmotic pressure (mmHg)	Osmolarity (mOsmol/L)
DL [b]	7.85	0.97	0.23	276
EMJH [a]	7.54	0.91	2.4	67
EMJH+ 130 mM NaCl	7.39	0.86	2.4	300
Plasma	7.40	1.35-1.85	28	296
NSS	5.50	1.02	0.25	308

[a] EMJH contains sodium phosphate dibasic and potassium phosphate monobasic buffering agents, bovine serum albumin (10% BSA).

[b] Compositions of dialysis solution are consisted of 0.1% glucose in a balanced electrolyte, bicarbonate-acetate solution.

Table S1: Compositions of plasma and dialysis fluid.

Compositions	Plasma	Dialysis fluid
Theoretical osmolarity (mosmol/L)	296	276
Bicarbonate-acetate	24	38
Sodium (Na^+)	142	138
Potassium (K^+)	4	3
Calcium (Ca^+)	2.5	3.5
Magnesium (Mg^+)	1.0	1.0
Chloride (Cl^-)	103	109.5
Glucose (mmol/L)	5-7	5.5
pH	7.4	7.7

Complete EMJH and 130 mM NaCl EMJH had viscosities at a shear rate of 150 s^{-1} of 0.91 and 0.86 cP, respectively. EMJH and 130 mM NaCl supplemented EMJH had a COP value of 2.4 mmHg, higher than NSS (0.9% saline solution) of 0.25 mmHg. The calculated

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osmolarity of 130 mM NaCl in EMJH was 300 mOsm/L similar to 296, and 308 mOsm/L of NSS and plasma, respectively (Table 1).

3.2. Development of in vivo mimic mammalian host model

When *Leptospira* was incubated at 37 °C for 24 h, the spirochete in 0.9% saline solution died, whereas the spirochete survived in EMJH and 130 mM NaCl EMJH media and in dialysate solution. Cell density and viability, however, were observed in 130 mM NaCl EMJH media culture at 30 °C. Leptospires recovered from a dialysis solution at 24 h post-inoculation were vigorously motile under dark-field microscopy. The methodology of preparation of secretome in dialysis solution has not been previously described and requires validation of secretomes contaminated with outer membrane protein by immunoblotting using anti-LipL32 serum [16] and colorimetric substrate. The sample showed positive LipL32 reactivity, indicating outer membrane proteins contaminated the secretome, and was excluded from the study. In this study, pathogenic *L. interrogans* serovar Icterohemorrhagiae cultured in EMJH media in the late log phase of growth at 30 °C was allocated to *in vivo* mimicking physiological mammalian conditions by incubating the leptospires in a physiological osmolarity dialysis solution at 30 °C (osmolarity shift), 37 °C (osmolarity and temperature shifts), and at 37 °C with 5% CO_2 (*in vivo*-like condition) for 24 h.

3.3. Analysis of the Leptospira RGA Secretomes

The CBB stained SDS-PAGE gel of *Leptospira* RGA secretomes revealed a unique secretome pattern ranging from 10–170 kDa compared to other serovars. Similar secretome patterns in response to osmolarity or temperature upshifts were observed (Figure 1A). There was no visual difference in the protein pattern of the secretome collected at 24 and 48 h post-incubation. The pH of the dialysis secretome increased from 7.7 to 8.5 at 30 °C and 37 °C, and 8.0 at 37 °C with a 5% CO_2 overlay.

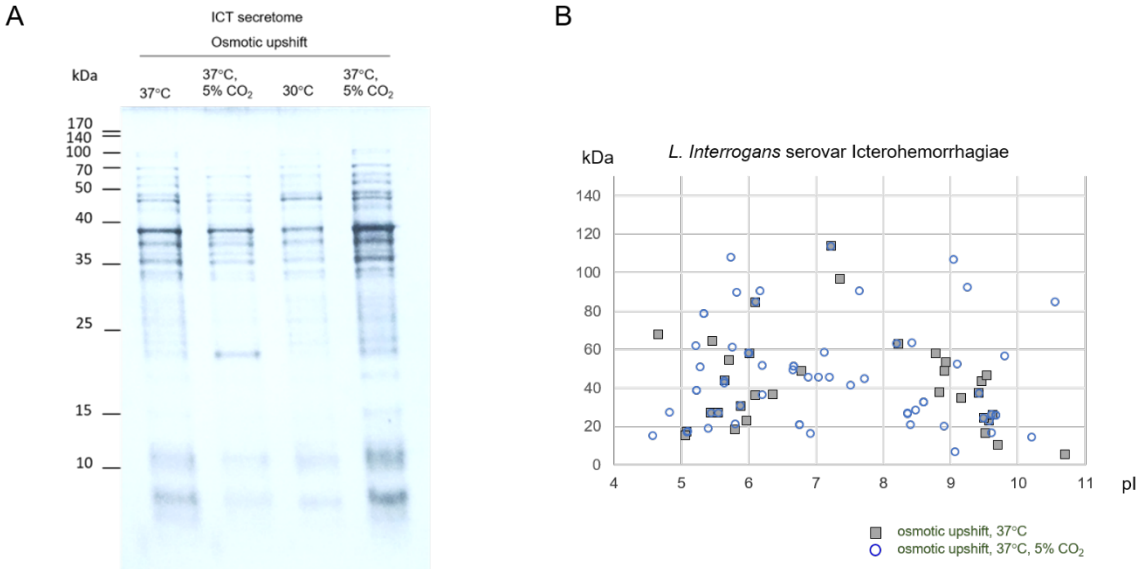

Figure 1. Analysis of *Leptospira* RGA secretomes.

Leptospira RGA secretome patterns were induced by physiological osmotic and temperature upshifts at 37 °C and 37 °C with 5% CO2 for 24 hours, concentrated, and analyzed using 13% SDS-PAGE, followed by CBB G250 stain. Secretome patterns ranged in molecular size from 10 to 180 kDa **(A).** Secreted proteins under osmotic and temperature upshifts displayed a range of protein molecular weight between 5.7-90.5 kDa and pI ranges of 4.5–7.5 and 8.0–10.6 **(B).**

Secreted proteins in the secretome in response to *in vivo* mammalian transitions including nutrient deprivation, osmolarity and temperature upshifts and stresses were

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identified. A MS database search revealed 44, 75, and 84 proteins matched to *Leptospira* proteins induced expression under osmotic upshift, physiological osmotic and temperature shifts, and *in vivo*-like conditions, whereas there were 9, 12, and 48 potential secreted proteins, respectively. The MW and the isoelectric point (pI) of the predicted secreted protein ranged from 5.7-90.5 kDa and 4.57-10.69, respectively (Figure 1B). The secreted proteins in response to osmotic and temperature shifts likely formed two clusters with respect to predicted (pI) values. The majority of the proteins belonged to the cluster with a pI of 4.5–7.5, while the other cluster was of proteins with a pI of 8.0–10.6.

Venn diagram of the secreted proteins of *Leptospira* RGA in response to osmotic shift (9 proteins), thermal stress (1 protein), osmotic and temperature shifts (9 proteins) and *in vivo* mammalian transition (48 proteins) was illustrated in Figure 2.

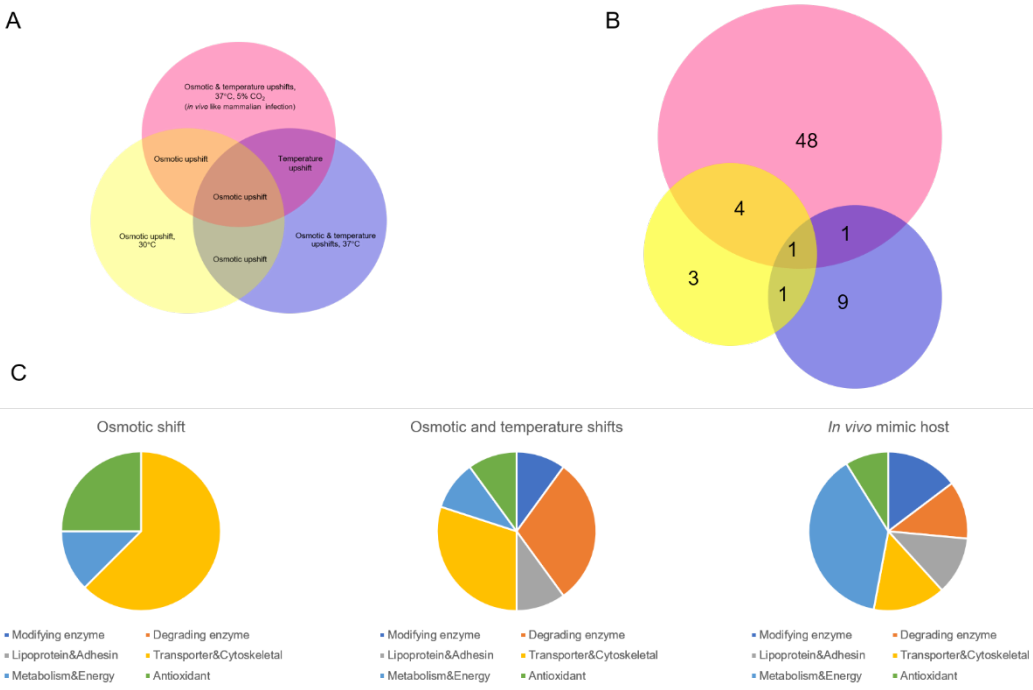

Figure 2. Secreted proteins of *Leptospira* RGA secretomes.

The Venn diagram of the numbers of the secreted proteins of the secreted proteins of *L. interrogans* serovar Icterohemorrhagiae str. RGA secretomes in response to physiological osmotic upshift at 30 °C (11 proteins), thermal stress (1 protein), physiological osmotic and temperature upshifts at 37 °C (12 proteins) and physiological osmotic and temperature upshifts at 37 °C with 5% CO_2 (48 proteins) **(A and B).** The percentage of secreted proteins subsets of each condition is categorized into modifying enzymes, degrading enzymes, metabolism and energy, lipoproteins and adhesins, transporters and cytoskeletal proteins, and hypothetical proteins **(C).**

3.4. Secreted Proteins in Response to Osmotic Stress

The transporters and porins expressed in response to physiological osmolarity were phosphate transporter and heavy metal efflux pumps, whereas heavy metal efflux, TonB-dependent receptor, and ompA family protein. Phosphate-selective porins O and P, an anion-specific phosphate selective porin, were identified in response to phosphate limitation and under salt- and glucose-induced osmotic stress. Under osmotic upshift, the OmpA family protein LA0056 was identified. The protein was responsible for peptidoglycan binding activity. Membrane cytoskeletal polymer-forming cytoskeletal family protein or putative cell shape determination protein involved in a cell division protein was found under osmotic stress and was downregulated expression when coregulated with thermal upshift (emPAI = 0.21 *vs* 0.06).

In addition, heat shock protein (chaperon GrpE), antioxidant (Redoxin), degradation enzyme (Glycosyl hydrolase), and metabolic enzyme (Ketol-acid reductoisomerase) were

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identified as secreted proteins induced by hyperosmotic stress. Glycoside hydrolase domain protein (LA0916) is a putative lactoylglutathione lyase involved in carbohydrate metabolism of the L-methylmalonyl-CoA matabolic process. This enzyme is involved in responding to glycan stress and was found to relate to survival. Ketol-acid reductoisomerase LA4242 (*ilvC*) is an enzyme oxidoreductase that catalyzes branch chain amino acid biosynthesis such as valine, leucine, and isoleucine. LA4242 is upregulated when the osmotic and temperature are both increased to 37 °C with 5% CO2 (emPAI = 0.09 vs 0.09 vs 0.18).

Non-secreted phospholipases, including putative lysophospholipase, were identified under osmotic upshift, and outer membrane phospholipase A was found under *in vivo* mammalian host.

3.5. Secreted Proteins from Leptospira RGA Secretome in Respond to Temperature Upshift

Proteins in response to temperature stress include lipoproteins LA2470 and interacting proteins, degradation enzymes including zinc metallopeptidase, Rhs family protein, thermolysin metallopeptidase, and α/β hydrolase family proteins (Table 2).Repeat domain proteins such as the LRR proteins LA0049, LA3725, and ankyrin-related protein LA0559 were discovered to be secreted proteins that responded to *in vivo* mammalian transition. Under heat stress, FG-GAP repeat protein (EQA54597.1) was found.

Table 2. Secreted proteins identified from *Leptospira* RGA secretomes in dialysis solution at 30 °C, 37 °C, and 37 °C, with 5% CO_2 for 24 h.

Accession no.	COG	emPAI[1]			Locus Tag[2]	Protein[3]
		30 °C	37 °C	37 °C, 5% CO_2		
Information storage and processing						
EMO27615.1	K	—	—	0.02	LA1807	ParB-like protein
EMN33155.1	L	—	—	0.02		Eco57I restriction-modification methylase
EMN52472.1	L	—	—	0.03	LA1854	ISXO2-like transposase domain protein
KPA32395.1	L	—	—	0.17	LA3725	Intein-C-terminal splicing domain protein
Cellular process and signaling						
WP_020766922.1	D	—	—	0.03	LA2980	Stage II sporulation protein E
EPG66159.1	D	0.24	—	0.06		Polymer-forming cytoskeletal family protein
EPG65797.1	M	0.07	—	—		Phosphate-selective porin O and P
ABZ93487.1	M	—	0.06	—		Heavy metal efflux pump
EQA62403.1	M/H	—	0.07	—	LA1674	Poly-gamma-glutamate synthase PgsB
EPE86531.1	M	—	0.13	—	L A3456	Peptidase, M23 family
ALO00286.1	M	—	0.13	—	L A1765	Rhs family protein/exotoxin
WP_000551773.1	M	—	—	0.06	LA0709	M23 metallopeptidase
EPG67955.1	M/U	0.06	—	0.06	L A3733	Outer membrane efflux protein
EPG64441.1	M/N/U	0.2	0.2	—	LA0056	OmpA family protein
ASV09925.1	N	—	—	0.05	LA4183	Endoflagellar motor protein
KPA32476	N/T	—	—	0.16	LA0049	Leucine rich repeat protein
EPG65127.1	O	0.13	—	—		Co-chaperone GrpE
EPG64449.1	O	0.16	—	—	LA0862	Redoxin
OOC00378.1	O	—	—	0.10	LA3705	Chaperone DnaK
EMO05204.1	O	—	—	0.16	LA2809	Peroxiredoxin
WP_00217710.1	T	—	—	0.03		Methyl-accepting chemotaxis protein
EMG10751.1	U	—	—	0.23	LA2319	Transport energizing protein, ExbD/TolR domain protein
EKR16759.1	V	—	—	0.08	LA2470	Putative lipoprotein
Metabolism						
EMO25988.1	C	—	—	0.07	LA0296	Crotonyl-CoA reductase
WP_004765857.1	C	—	—	0.15	LB327	Aconitate hydratase
EIE01589.1	C	—	—	0.13		Succinate dehydrogenase cytochrome β subunit, β558 family
EMY06802.1	E	—	—	0.03		Thermolysin metallopeptidase, α-helical domain protein
ASV09144.1	E	—	—	0.05	LA0042	GMC oxidoreductase
WP_020765520.1	E	—	—	0.22	LA1409	Serine hydroxymethyltransferase
EOQ87745.1	E	—	0.24	—	LA3312	Glyoxalase-like domain protein
ABJ77767.1	E/H	0.09	0.09	0.18	L A4242	Ketol-acid reductoisomerase
WP_020765929.1	G	—	—	0.09	LA0439	Phosphoglyceromutase
ABZ95019.1	F	—	—	0.12		Uridylate kinase
EMJ95366.1	H	—	—	0.04	LA3114	NAD(P)-binding Rossmann-like domain protein
WP_01785147.1	H	—	—	0.16	LA3317	Dihydrofolate reductase
EIE00417.1	I	—	0.09	—		α/β hydrolase family protein
ASV08569.1	I	—	—	0.07	LA4159	ACP S-malonyltransferase
WP_036025616.1	I	—	—	0.09	LA1345	α/β hydrolase

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Accession no.	COG	emPAI[1]			Locus Tag[2]	Protein[3]
		30 °C	37 °C	37 °C, 5% CO_2		
WP_002177513.1	I	—	—	0.09	LA2501	Putative α/β hydrolase
ASP42974.1	I	—	—	0.17	LA3627	Acyl-CoA dehydrogenase
WP_025176825.1	I	—	—	0.2	LA0899	MaoC family dehydratase
ASV05635.1	P	—	—	0.04	LA3102	TonB-dependent receptor
EQA72152.1	Q	—	0.06	0.06	LA0379	Methyltransferase
				Poorly characterized		
ABJ77150.1	R	—	—	0.08	LA0559	Ankyrin-related protein
WP_061244535.1	R	—	—	0.03	LA1299	Ankyrin repeat-containing protein
EQA53780.1	S	0.07	—	—	LA0916	Glycoside hydrolase domain protein
ASV08671.1	S	—	—	0.21	LA0343	Aldehyde-activating protein
EQA54597.1		—	0.05	—		FG-GAP repeat protein
				Hypothetical proteins		
EMN99309.1		—	—	0.06	LA3490	PF07598 family protein
KPA28575.1		—	—	0.18	—	Uncharacterized AMR48_1395
AAS71202.1		—	—	0.18	LA1015	Conserved hypothetical protein
EQA62912.1		0.06	—	0.06	LA1312a	Hypothetical protein
EMY76193.1		0.1	—	0.1	LA1728	Hypothetical protein
WP_061234367.1		—	—	0.05	LA0365	Hypothetical protein
WP_036027338.1		—	—	0.15	LA1965	Hypothetical protein
WP_082271210.1		—	—	0.21	LA2342	Hypothetical protein
EMN50376.1		—	—	0.05	LA0811	Hypothetical protein
EMJ53969.1		—	—	0.05	LA1965	Hypothetical protein
EKR08169.1		0.63	—	—		Hypothetical protein
EMY68008.1		—	0.03	—		Hypothetical protein
EOQ97649.1		—	0.05	—		Hypothetical protein
WP_002178239.1		—	—	0.05		Hypothetical protein
WP_061243674.1		—	—	0.12		Hypothetical protein
ASP41581.1		—	—	0.18		Hypothetical protein
EMO30130.1		—	—	0.22		Hypothetical protein
WP_080021233.1		—	—	0.32		Hypothetical protein
EMO26226.1		—	—	0.4		Hypothetical protein
EKR24801.1		—	—	0.5		Hypothetical protein
WP_000313466.1		—	—	0.05		Hypothetical protein

[1] Exponentially modified protein abundance index (emPAI) value for estimation of absolute protein amount in the proteomics. —, no protein.

[2] Putative ortholog present in *L. interrogans* serovar Lai strain 56601 based on a BlastP alignment, threshold and minimum amino acid sequence identity over 80%.

Polypeptides containing ≤100 amino acids are considered to be small proteins.

COG functional categories are as follow: J, translation, including ribosome structure and biogenesis; L, replication, recombination and repair; B, chromatin structure and dynamics; C, Cellular processes and signaling: D, cell division and chromosome partitioning; V, defense mechanism; O, posttranslational modification, protein turnover, chaperones; M, cell wall structure and biogenesis and outer membrane; U, intracellular trafficking, secretion and vesicular transport; Metabolism transport and metabolisms: C, energy production and conversion; G, carbohydrate metabolism and transport; E, amino acid metabolism and transport; Metabolism: P, inorganic ion transport and metabolism; S, function unknown.

Table S2: Potential moonlighting proteins identified from *Leptospira* RGA secretomes.

Accession no.	Locus Tag	COG	emPAI	Protein	Moonlighting function
WP_017851519.1	LA1879	O	0.07	Chaperone ClpB	Metabolic process
EMO05204.1	LA2809	O	0.52	Peroxiredoxin	Plasminogen binding
OOC00378.1	LA3705	O	0.10	DnaK/HSP70	Plasminogen binding
EPG64449.1		O	0.16	Redoxin	Plasminogen binding
WP_004754792.1	LA0313	J	0.17	Ef-G	adhesin
EMO26793.1	LA0737	J	0.14	Ef-Tu	Plasminogen binding Fibronectin binding
EMO28780.1	LA3297	J	0.14	Ef-Ts	Plasminogen binding
EMO28561.1	LA1240	J	0.09	Threonine-tRNA ligase-like protein	Promote vascular development
WP_000551773.1	LA0709	L	0.02	M23 metallopeptidase	Plasminogen binding Fibronectin binding
EMO00242.1	LA3456	L	0.02	Peptidase, M23 family	Fibronectin binding
WP_004765857.1	LB327	C	0.15	Aconitate hydratase	RNA-binding
APH43078.1	LA4171	C	0.06	Isocitrate dehydrogenase	mRNA binding
EMO25988.1	LA0296	C	0.07	Crotonyl-CoA reductase	ECM binding

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Accession no.	Locus Tag	COG	emPAI	Protein	Moonlighting function
OOB94044.1	LA2139	C	0.09	Malate dehydrogenase	mRNA binding
WP_020765520.1	LA1409	E	0.22	Serine hydroxymethyltransferase	mRNA-binding
ALO00256.1	LA1719	E	0.1	Cysteine synthase A	Transcriptional regulator
Q8EYH2	LA4242	E	0.18	Ketol-acid reductoisomerase type1	Antioxidant
WP_020766873.1	LA1703	G	0.43	Phosphoglycerate kinase	Plasminogen binding
EMN49773.1	LA353	G	0.06	Pyruvate kinase	Adhesion
WP_01785147.1	LA3317	H	0.15	Dihydrofolate reductase	RNA-binding

M23 family zinc dependent metalloprotease (LA0709, LA3456), thermolysin metallopeptidase, and α-helical domain protein (EMY06802.1) were identified under *in vivo* mammalian conditions. In addition, membrane-bound metalloendopeptidase (zinc protease) without signal peptide was identified, including putative RIP metalloprotease, RseP (LA3628) and ATP-dependent protease HslVu ATPase subunit protein under temperature shift. The α/β hydrolase LA1345, putative α/β hydrolase LA2501 (*mhpC*) and non-secreted metallo-β-lactamase domain protein were identified under osmotic and temperature shifts (Table 3).

Table 3 Potential proteases identified in the *Leptospira* RGA secretomes.

Protein	Secretion	Condition
Proteases		
Peptidase, M23 family LA3456	Yes	37 °C, 5% CO_2
M23 metallopeptidase LA0709	Yes	37 °C, 5% CO_2
Peptidase, M23 family	No	37 °C
Thermolysin metallopeptidase, α- helical domain protein	Yes	37 °C, 5% CO_2
ATP-dependent protease HslVu ATPase subunit protein	No	37 °C
Peptidase M16 inactive domain protein	No	37 °C
Putative RIP metalloprotease, RseP	No	37 °C, 5% CO_2
Hydrolases		
α/β hydrolase	Yes	37 °C, 5% CO_2
Putative α/β hydrolase	Yes	37 °C, 5% CO_2
Glycoside hydrolase domain protein	Yes	30 °C
Metallo-β-lactamase domain protein	No	37 °C

3.6. Secreted Proteins from Secretome in Respond to Oxidative stress

Oxidative stress response proteins induced by osmolarity and temperature upshifts consisted of antioxidants including heat shock proteins HSP70/DnaK (LA3705), co-chaperon GrpE (LA3705), antioxidants, i.e., Thiol-specific redoxin (LA0862), alkyl hydroperoxide such as peroxiredoxin (LA2809), and MaoC family dehydratase (LA0899).

Energy and metabolisms proteome was induced under *in vivo* like model (Table 1) include energy production (COG:C) such as crotonyl-CoA reductase, aconitate hydratase, succinate dehydrogenase cytochrome b subunit, β558 family; amino acids metabolism and transport (COG:E) such as GMC oxidoreductase, ketol-acid reductoisomerase type1, and serine hydroxymethyltransferase; lipid metabolism and transport (COG:I) such as ACP S-malonyltransferase, α/β hydrolase, acyl-CoA dehydrogenase, and MaoC family dehydratase, coenzyme transport and metabolism such as NAD(P)-binding Rossmann-like domain protein, dihydrofolate reductase (*folA*) LA3317. In addition, several moonlighting proteins were categorized into the energy and metabolism group (COG:C, E, G, H) (Table S1).

3.7. Moonlighting Proteins Identified from Leptospira RGA Secretome

Moonlighting proteins (Table S1) were identified from the proteome in response to *in vivo* mammalian infection, including secreted and non-secreted housekeeping enzymes in protein synthesis, glycolysis pathway, citric acid cycle, energy production, amino acid

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metabolisms, and chaperones. Several proteins exhibited secondary moonlighting functions involved in plasminogen-binding activity, including peroxiredoxin, chaperon DnaK/HSP70, redoxin, Ef-Ts (LA3297), and phosphoglycerate kinase. Proteins exhibited moonlighting plasminogen and fibronectin-binding activity, including Ef-Tu (LA0737), and M23 metallopeptidase. mRNA binding moonlighting proteins were identified, including aconitate hydratase, malate dehydrogenase, serine hydroxymethyltransferase, and dihydrofolate reductase. Among them, peroxiredoxin, phosphoglycerate kinase, and serine hydroxymethyltransferase were highly induced as shown by emPAI values of 0.52, 0.43, and 0.22, respectively.

Potential moonlighting proteins were categorized into COG: O, J, L, C, E, G, H. Indeed, moonlighting proteins classified as COG:O included the chaperone ClpB, peroxiredoxin, DnaK/HSP70, redoxin, and Ts; moonlighting proteins classified as COG:L included M23 metallopeptidase; and metabolism group includes COG:C, which includes aconitate hydratase, isocitrate dehydrogenase, crotonyl-CoA reductase. These proteins contributed to moonlighting functions involving transcriptional regulators and signaling, plasminogen, and fibronectin adhesion.

3.8. Exploration of the Immunogenicity-and Virulent-associated of Leptospira RGA secretome

Induced immunogenic proteins induced upon osmotic shift, osmotic and temperature shifts, and *in vivo* mammalian infection were revealed to have similar IgG reactive bands approximately at 35 kDa by using anti-*Leptospira* immune serum (Figure 3). Several secreted proteins induced in response to oxidative stress were identified as potential immunogenic proteins, including chaperone DnaK (LA3705), peroxiredoxin (LA2809), endoflagellar motor protein (LA4183), succinate dehydrogenase cytochrome β subunit, β558 family, methyltransferase, and hypothetical LA0365 protein. Moonlighting immunogenic proteins were also identified from *Leptospira* RGA proteomes under *in vivo* mammalian infection, such as adenylate/guanylate cyclase, protein synthesis elongation factor G (Ef-G), translation elongation factor Tu (Ef-Tu), and Ef-Ts.

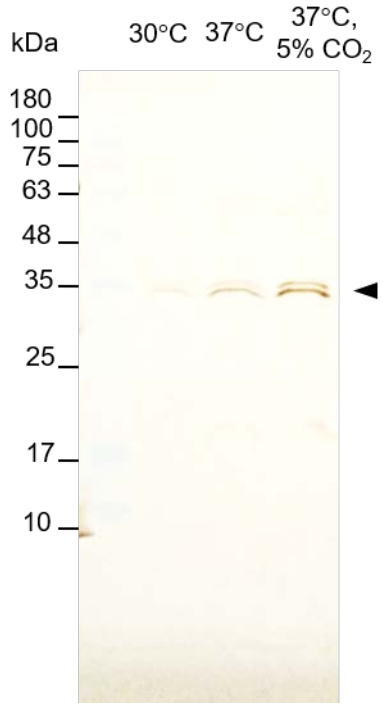

Figure 3. Determination of immunogenic proteins patterns in *Leptospira* RGA secretomes.

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Activation of immunogenic proteins at 35 kDa was revealed in *L. interrogans* serovar Icterohemorrhagiae str. RGA secretomes were induced by physiological osmolarity at 30 °C, 37 °C, and 37 °C with 5% CO_2 for 24 h by anti-*Leptospira* immune serum, followed by anti-mouse IgG-HRP and DAB substrate.

Of 25 potential virulent determinants (Table S3) were analyzed from *Leptospira* RGA secretomes by manually searching for their associations with virulence activities in other species. Potential virulent-candidates were functionally involved in transporters, chaperones, antioxidants, proteases, lipoprotein, flagellin, and repeat domain proteins.

Table S3: Potential virulent factors identified from *Leptospira* RGA culture supernatant.

No.	Accession no.	COG	Secreted protein	Locus Tag	Protein
1	EMO00242.1	M	No	LA3456	Peptidase, M23 family
2	WP_000551773.1	M	Yes	LA0709	M23 metallopeptidase
3	ALO00286.1	M	Yes	LA1765	Rhs family protein
4	WP_000313035.1	M	No		TolC family protein
5	EMO028805.1	M	No		Efflux ABC transporter, permease protein
6	KPA32476	N/T	Yes	LA0049	Leucine rich repeat protein
7	ALO00811.1	N	No		Endoflagella filament core protein
8	ASV09925.1	N	No	LA4183	Endoflagellar motor protein
9	WP_002177423.1	N	No		Flagellin
10	EMO05204.1	O	Yes	LA2809	Peroxiredoxin
11	OOC00378.1	O	Yes	LA3705	Molecular chaperone DnaK
12	WP_017851519.1	O	No	LA1879	ATP-dependent chaperone ClpB
13	WP_00217710.1	T	Yes		Methyl-accepting chemotaxis protein
14	EMN09781.1	V	No	LA2470	Putative lipoprotein
15	WP_082271210.1	V	No		Lipoprotein
16	WP_000551773.1	L	Yes		Hypothetical protein
17	gi|384115331	C	Yes		succinate dehydrogenase cytochrome β subunit, β558 family
18	PQL61708.1	C	No		Toxin-antitoxin system HicB famiy antitoxin
19	EMY06802.1	E	Yes		Thermolysin metallopeptidase, α- helical domain protein
20	WP_036025616.1	I	Yes	LA1345	α/β hydrolase
21	WP_002177513.1	I	Yes	LA2501	α/β hydrolase
22	WP_025176825.1	I	Yes	LA0899	MaoC family dehydratase
23	ASV05635.1	P	Yes	LA3102	TonB-dependent receptor
24	WP_061244535.1	R	Yes	LA1299	Ankyrin repeat-containing protein
25	ABJ77150.1	R	Yes	LA0559	Ankyrin-related protein

3.9. Hypothetical Proteins and Protein Family Proteins in Leptospira RGA Secretome

Hypothetical proteins presenting orthologous proteins of *L. interrogans* serovar Lai including hypothetical LA1312a and LA1728, were identified under physiological osmotic upshift. Hypothetical LA1312a contains a HEAT repeat domain. Hypothetical proteins induced in response to *in vivo* like mammalian transition were annotated LA1015, LA0365, LA1965, LA2342, LA0811, and LA1965 (emPAI of 0.05-0.21), and another 8 unique hypothetical proteins with emPAI ranging from 0.05-0.5. PF07598 family protein (LA3490) was also identified under mammalian host infection. This protein sequence was conserved among pathogenic *Leptospira* spp.

4. Discussion

During natural and experimental infections in humans and reservoirs, leptospires rapidly disseminate homogeneously to the kidneys to colonize and persist within renal tubules. To assess virulent-associated determinants in the secretome in response to physiological osmolarity and temperature shifts, an *in vivo* mimic condition was established to study the secretome in the early stage of infection or during transition from the environment into a mammalian host by incubating *Leptospira* RGA in a protein-free, 0.1% glucose bicarbonate-acetate dialysis solution that contained glucose, ions, small molecules, and an

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osmolarity close to plasma. This model is similar to the dialysis membrane chamber (DMC) model [30], which leptospires are likely to encounter within the proximal convoluted tubules.The *in vivo* mimic model was incubated at 30 °C (osmolarity shift), 37 °C (osmolarity and temperature shifts), or at 37 °C with 5% CO_2 (*in vivo*-like condition) for 24 hours. Hemodialysis solution is used for extracorporeal blood purification such as hemodialysis and peritoneal analysis, renal replacement therapy, and acute kidney injury [31]. This fluid has thermoregulation, osmoregulation, and hemostatic equilibrium. We also provided a 5% CO_2 overlay in a cell suspension to maintain pH homeostasis in an *in vivo*-like model. LipL32 outer membrane lipoprotein as a biomarker for validating outer membrane contamination in the secretome due to protein abundance and its constant expression under a wide-range of growth conditions, temperature, osmolarity, and/or exposure to serum [21,32,33]. Classical secreted proteins containing N-terminal signal sequences, i.e. Sec/SpI secretory signal peptide, or Tat/SpI signal peptide, and non-classical secreted proteins were verified from secretomes and were classified into functional COG.

During infection in a mammalian host, pathogenic leptospires transition from environmental to host conditions such as changes in temperature [33], osmolarity [11], serum exposure [21]. and macrophage interactions [34]. Pathogens respond to environmental changes by altering gene expression of leptospiral exoproteins involved in motility, signal transduction, and energy production [35,36]. In this study, the late log-phase of leptospires cultured in EMJH media was transitioned to physiological osmotic dialysis solution. They require essential nutrients, including thiamine, biotin, calcium, magnesium, iron, and phosphate for growth. Under physiological osmolarity transition by glucose and sodium in protein-free dialysis solution, phosphate transporters, heavy metal efflux transporters, and antioxidants were investigated. Phosphate-selective porins O and P are anion-specific porins or bacterial phosphate-selective porins that are identified under osmotic stress. The porin is specific to phosphate and higher than chloride ions. The pore is overexpressed under phosphate-starvation during the stationary growth phase.

OmpA protein LA0056 is a multifunctional protein involved in transport of biopolymers, peptidoglycan binding activity in membrane biogenesis, motility, adhesion, and evasion of host defense and stimulates host inflammatory cytokines. The outer membrane efflux protein is a TolC family outer membrane protein for efflux transporter activity. It acts as a virulent determinant, found under physiological upshift of osmotic and temperature. The heavy metal efflex pump identified under temperature upshift is a cation-transmembrane transporter and is important for resistance to cobalt, zinc, and cadmium [37]. The TonB-dependent receptor LA3102 contains the OM-channels superfamily and the biopolymer transport protein ExbD, which are both involved in TonB-dependent energy-dependent transport of various receptor-bound substrates. The TonB-dependent receptor is an iron-siderophore transporter that uses energy from the TonB, ExbB, and ExbD inner membrane complexes.

We identified non-secreted signal transduction machinery under osmotic upshift such as adenylate/guanylate cyclase catalytic domain protein, cyclic diguanylate phosphodiesterase (EAL) domain protein, response regulator receiver domain protein, and nitrogen regulatory protein P-II.

In vitro, the transition to physiological osmolarity appears to be associated with a shift from degradation to biosynthesis of fatty acids. Genes encoding acyl carrier protein synthase, which is necessary for fatty acid biosynthesis, were upregulated by sodium chloride [11]. Consistent with our study, we identified secreted ACP S-malonyltransferase and non-secreted fatty acid/phospholipid synthesis proteins under osmotic upshift. Growth at physiological osmolarity *in vivo* may require carbon sources that are absent in EMJH medium, as *L. interrogans* down-regulates its fatty acid catabolism pathway [11]. Under physiologic osmolarity by increased sodium chloride in EMJH media, signal transduction (COG: T) in adaptation to host conditions and lipoproteins were up-regulated. Osmotic and general stress genes were not induced [11]. *Leptospira interrogans* uses long-chain fatty acids as its sole carbon source *in vitro* lipid composition of the membrane and LPS may be

altered at physiological temperature due to several enzymes in lipid metabolism, including acyl-CoA dehydrogenase homolog involved in beta-oxidation of fatty acids and epimerase homolog. A cytoplasmic glycosyltransferase homolog was found in the secretome of *Leptospira* Icterohemorrhagiae under physiological temperature upshifts, while these two enzymes were downregulated in EMJH culture at physiological temperature upshift [10]. Glycosyltransferase moonlights as a transcription regulator of motility gene transcription. MaoC-family dehydratase or enoyl-CoA homolog is involved in cholesterol side chain degradation and was found under *in vivo* like conditions. Acyl dehydratase is a potential virulent factor of *Leptospira*, however, genes involved in lipid metabolism (COG: I) were upregulated in virulence-attenuated *Leptospira* strains [38].

Oxidative stress response proteins induced by osmolarity and temperature upshifts consisted of antioxidants, including heat shock proteins and antioxidants. *Leptospira* displays cytochrome C, oxidase, and peroxidase (catalase), which are required for *in vivo* leptospiral survival by mediating protection against reactive oxygen species [36]. LA2809 encodes peroxiredoxin, which takes part in the bacterial antioxidant defense [39]. The GrpE forms a chaperone system of DnaK/DnaJ/GrpE and ClpB systems for reactivation of the stress-aggregated proteins and promotes bacterial survival under oxidative and thermal stresses. *In vivo* ATP-dependent chaperone ClpB was found in culture supernatant under *in vivo* mammalian conditions. ClpB is one of the *L. interrogans* factors that is necessary for defense against oxidative stress. ClpB belongs to the HSP100/Clp subfamily of the AAA+ ATPase which is conserved among prokaryotic pathogens and plants. This chaperone, ClpB, is one of the targets for diagnosis and therapy of drug-resistant bacteria.

Analyses of *Leptospira* genomes have shown that pathogenic *Leptospira* possess a large number of genes encoding putative extracellular proteins containing leucine-rich repeat (LRR) and ankyrin repeat (ANK) domains and are involved in mediating host-cell attachment and invasion, but their functions remain unknown in *Leptospira.* One of the promising diagnostic targets is the leucine rich repeat (LRR) protein. *Leptospira interrogans* serovar Lai genome possesses a number of genes encoding putative extracellular secreted proteins containing LRR and ANK domains of about 2.6%. In this study, leucine rich repeat proteins LA0049, LA3725, ankyrin related protein LA0559, and FG-GAP repeat protein (EQA54597.1) were identified as responding to physiological osmotic and temperature upshifts.

Moonlighting proteins have a canonical biochemical function inside the cells, such as enzymes and chaperones, and perform a second biochemical function on the cell surface, so-called exported proteins or secreted proteins. These multifunctional proteins play crucial roles in physiological processes but have been shown to contribute to infection by many pathogens [40]. Several moonlighting proteins were identified as similar to those previously identified by [36,41]. A diverse array of ECM-binding proteins has been identified in the *Leptospira* genome suggesting a redundancy of adhesion molecules that are probably part of the invasion strategies of *Leptospira*. Indeed, many putative adhesins bind to fibronectin, laminin, collagen, mucin, or act as surface receptors for soluble protein binding and play a role in infection and virulence. These are moonlighting proteins that exhibit secondary moonlighting functions involved in plasminogen-binding. They include peroxiredoxin, DnaK/HSP70, redoxin, Ef-Ts, phosphoglycerate kinase, and plasminogen and fibronectin-binding activity, including Ef-Tu, and M23 metallopeptidase. Among them, peroxiredoxin, phosphoglycerate kinase, and serine hydroxymethyltransferase were found to be highly expressed proteins as shown by emPAI values of 0.52, 0.43, and 0.22, respectively. Ef-Tu can act as an adhesive moonlighting protein that binds to fetuin, heparin, plasminogen, vitronectin, laminin, and fibronectin. Translation Ef-Tu from *Streptococcus gordinii* has been found to bind to host mucin.

Cytoplasmic enzymes have evolved to serve as moonlighting transcriptional or translational regulators that bind DNA or RNA, respectively, including cysteine synthase A, dihydrofolate reductase, malate dehydrogenase, and serine hydroxymethyltransferase. Aconitate hydratase catalyzes the isomerization of citrate to isocitrate in the citric acid

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cycle. When cellular iron is depleted, the aconitase changes conformation due to the loss of iron—sulfer cluster and moonlighting bind to the RNA to regulate expression of iron uptake protein. The moonlighting function of DHFR also binds to DHFR mRNA to regulate protein expression. Cytoplasmic threonine-tRNA ligase-like proteins play a role in cancer development from the secreted form by promoting vascular development. *L. interrogans* immunogenic proteins can elicit a host immune response as the immunogenic proteins are recognized by specific antibodies in the immune serum or by serum of infected subjects. The outer membrane proteins have been experimentally confirmed to be immunogenic proteins and have been developed for human and animal leptospirosis diagnosis, such as LipL21, LipL31, LipL32, LipL36, LipL41, Loa22, OmpL1, LigA, Loa22, LigB, Lsa63, GroEL, DnaK, and ompL1 [22,42-47]. Several secreted proteins under oxidative stress under *in vivo* conditions were identified as immunogenic proteins, including molecular chaperone DnaK (LA3705), peroxiredoxin (LA2809), hypothetical protein LA0365, endoflagellar motor protein (LA4183), succinate dehydrogenase cytochrome β subunit, β558 family. Immunogenic proteins include heat shock proteins HSP60/GroEL and HSP70/DnaK, LipL41, LipL32, and LipL45, ompL1.

Bacteria express a number of heat shock proteins (HSPs) such as molecular chaperones, including DnaK and GroEL, that could be found in the *Leptospira* secretome. When exported to the cell surface or secreted, they express on the intracellular/surface or intracellular/secreted moonlighting protein and exhibit more than one biochemical or biophysical function as a result of conformational change or post-translational modification [40].The HSP70/DnaK chaperone serves as a cell surface receptor for plasminogen in many species, including *Neisseria, M. tuberculosis*.

Moonlighting proteins such as protein synthesis elongation factor G, translation elongation factor Tu (Ef-Tu), Ef-Ts, and hypothetical LA0365 protein were identified as immunogenic antigen. Antibodies against Ef-Tu have been detected in a range of natural infections such as *K. pnuemoniae* infection. Recombinant Ef-Tu from *M. ovipneumoniae* was identified as a potent immune-stimulatory effector, induces an immune response in mice, stimulate cytokines and has shown a vaccine candidate potential.

Bacterial virulence determinants, functional proteins associated with virulence and their immunogenic secreted proteins have been extensively identified and characterized in order to identify potential diagnostic biomarkers, protective vaccine candidates and also therapeutic components against bacterial infections [15,16,48,49]. Fraser and Brown (2017) demonstrated that increased temperature and oxidative stress can trigger the response regulator mode of virulence genes during infection by induction of virulence-associated genes such as *lipl32, lipl41, lipl45, ompL1, sph2, lenA, ligB, loa22*. These annotated lipoproteins have been shown to be surface-exposed, located or associated with the outer membrane and an essential virulent factor [51]. Under temperature upshift and *in vivo* physiological conditions, potential secreted outer membrane lipoproteins such as Loa22, LigB, LipL45, LipL41 were not detected in our secretome samples. Protein may be lost during sample preparation, not amenable to identification by MS and/or may be not expressed or expressed at low levels below the limit of detection. However, we can identify many lipoproteins secreted by other serovars such as Autumnalis with an emPAI ranging from 0.05-0.25 (data not shown). PF07598 gene family has been described in unrelated α-proteobacteria species. Twelve members of the leptospiral PF07598 gene family were analyzed for *in vivo* expression in hamsters infected with virulent serovar Lai 55601 [52].

Under physiological osmotic and temperature changes, *L. interrogans* secrete proteases that degrade extracellular matrix and host plasma proteins. Production of proteases might be an important virulence determinant since culture-attenuated or saprophyte *Leptospira* did not show proteolytic activity to ECM or plasma proteins [41]. *Leptospira* serovar Icterohemorrhagiae secreted hydrolases under physiological osmotic upshifts such as glycosyl hydrolase LA0916 superfamily, i.e., glucosidase, xylosidase, and under osmotic and temperature upshifts such as putative α/β hydrolase family LA1345, LA2501 (*mhpC*) con-

tributed to protease, lipase, peroxidase, and esterase suggest virulent of the isolate. Membrane-bound metallo-β-lactamase (MBL) hydrolase under osmotic and thermal stress is a zinc metallohydrolase (glyoxalase) that catalyzes the hydrolysis of lactoyl glutathione. *Leptospira* serovar Icterohemorrhagiae secreted zinc-dependent peptidase family M23 contains endopeptidases that lyse bacterial cell wall peptidoglycans. Leptallo I, a protease that belongs to the M23 family, displays proteolytic activity against elastin. Leptallo I was shown to be secreted into the culture medium during leptospiral growth, and the protease could be recognized by leptospirosis IgG antibodies [53]. Under physiological osmotic and temperature changes, a protein from the Rhs family was discovered to be involved in the secretion of bacterial toxin.

5. Conclusion

In vivo mammalian model under physiological osmolarity and temperature transitions was successfully established in a protein-free dialysis solution. A dataset of secreted proteins of *Leptospira interrogans* serovar Icterohemorrhagiae secretomes under nutrient deprivation and under physiological mammalian transition which resembles the renal proximal tubular environment was identified. An overview of secreted proteins will supplement our understanding of *Leptospira* biology and pathogenesis during infection and also in response to environmental stimuli and their potential virulent determinants have the potential for developing leptospirosis vaccines and diagnosis.

Author Contributions:

Conceptualization, O.R. and S.M.; methodology, O.R., W.P., D.L., M.V., P.P., U.B., N.S., S.A., P.A., U.C., and S.M.; investigation, T.T., O.R., W.P., S.C., M.O., P.P., U.B., N.S., S.A., P.A., U.C., and S.M.; validation, O.R., W.P., S.M.; formal analysis, T.T., O.R., S.C., M.V., P.P., U.B., N.S., S.A., P.A., U.C., and S.M.; writing—original draft preparation, O.R. and S.M.; writing—review and editing, T.T., O.R., W.P., D.L., S.C., M.O., M.V., P.P., U.B., N.S., S.A., P.A., U.C., and S.M. All authors have read and agreed to the published version of the manuscript.

Conflict of Interests:

The authors have declared that they have no competing interests.

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Figure Legend

1. **Figure 1.** Analysis of *Leptospira* RGA secretomes.
   Leptospira RGA secretome patterns were induced by physiological osmotic and temperature upshifts at 37 °C and 37 °C with 5% CO2 for 24 hours, concentrated, and analyzed using 13% SDS-PAGE, followed by CBB G250 stain. Secretome patterns ranged in molecular size from 10 to 180 kDa **(A).** Secreted proteins under osmotic and temperature upshifts displayed a range of protein molecular weight between 5.7-90.5 kDa and pI ranges of 4.5–7.5 and 8.0–10.6 **(B).**
2. **Figure 2.** Secreted proteins of *Leptospira* RGA secretomes.
   The Venn diagram of the numbers of the secreted proteins of the secreted proteins of *L. interrogans* serovar Icterohemorrhagiae str. RGA secretomes in response to physiological osmotic upshift at 30 °C (11 proteins), thermal stress (1 protein), physiological osmotic and temperature upshifts at 37 °C (12 proteins) and physiological osmotic and temperature upshifts at 37 °C with 5% CO_2 (48 proteins) **(A and B).** The percentage of secreted proteins subsets of each condition is categorized into modifying enzymes, degrading enzymes, metabolism and energy, lipoproteins and adhesins, transporters and cytoskeletal proteins, and hypothetical proteins **(C).**
3. **Figure 3.** Determination of immunogenic proteins patterns in *Leptospira* RGA secretomes.
   Activation of immunogenic proteins at 35 kDa was revealed in *L. interrogans* serovar Icterohemorrhagiae str. RGA secretomes were induced by physiological osmolarity at 30 °C, 37 °C, and 37 °C with 5% CO_2 for 24 h by anti-*Leptospira* immune serum, followed by anti-mouse IgG-HRP and DAB substrate.