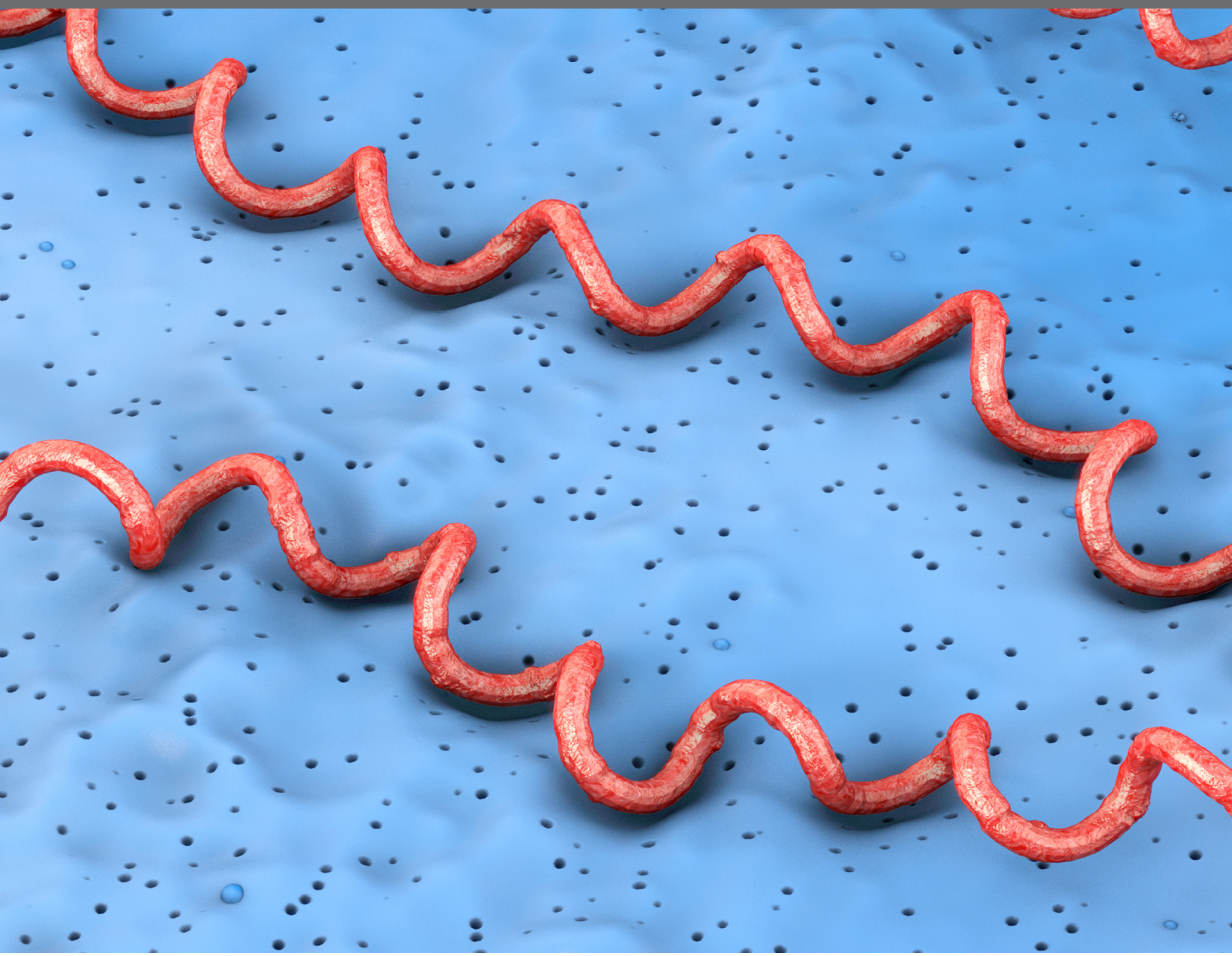
PATHOGENESIS OF *LEPTOSPIRA*

EDITED BY: Elsio A. Wunder Jr., Azad Eshghi and Nadia Benaroudj
PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-649-9
DOI 10.3389/978-2-88945-649-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

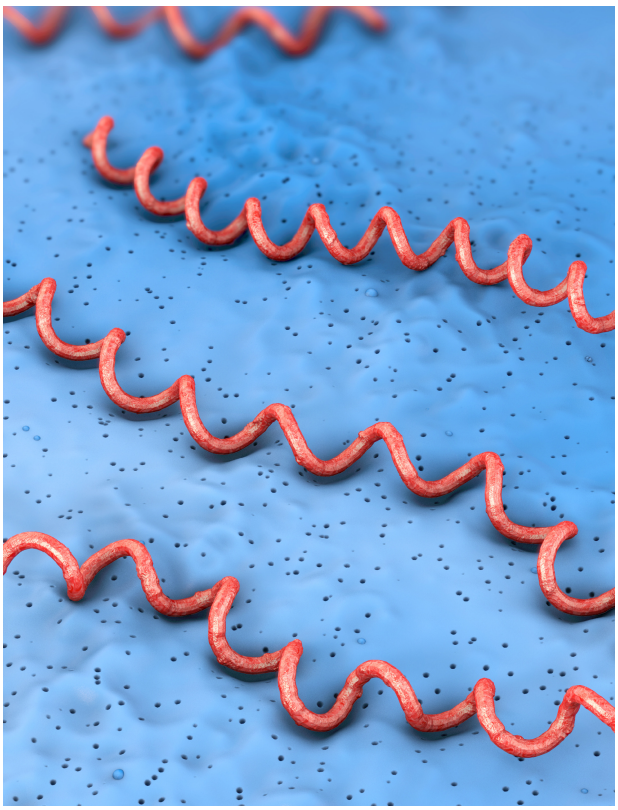
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

PATHOGENESIS OF *LEPTOSPIRA*

Topic Editors:
Elsio A. Wunder Jr., Yale School of Public Health, United States
Azad Eshghi, UVic Genome BC Protein Centre, Canada
Nadia Benaroudj, Institut Pasteur, France



Cover image: Christoph Burgstedt/Shutterstock.com

The present eBook, consisting of a compilation of research and review articles, focuses on the features and mechanisms adopted and explored by pathogenic leptospires to successfully establish infection in the host. Additionally, this eBook provides information to support future work focused on the development of new prevention approaches against this important yet neglected zoonotic disease.

Citation: Wunder Jr., E. A., Eshghi, A., Benaroudj, N., eds. (2018). Pathogenesis of *Leptospira*. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-649-9

Table of Contents

IV. PATHOGEN

frontiers in Cellular and Infection Microbiology

EDITORIAL
published: 11 September 2018
doi: 10.3389/fcimb.2018.00322

Editorial: Pathogenesis of *Leptospira*

Elsio A. Wunder Jr.[1]*, *Azad Eshghi*[2]* and *Nadia Benaroudj*[3]*

[1] Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, United States, [2] UVic Genome BC Protein Centre, Victoria, BC, Canada, [3] Biology of Spirochetes Unit, Institut Pasteur, Paris, France

Keywords: ***Leptospira***, **leptospirosis, pathogenesis, virulence, regulation, immune response**

Editorial on the Research Topic

Pathogenesis of *Leptospira*

Leptospira are a diverse group of spirochete bacteria classified into 13 pathogenic species and >300 serovars (Picardeau, 2017; Thibeaux et al., 2018). *Leptospira* are the etiological agents of leptospirosis, a neglected life-threatening disease that occurs in a diverse range of epidemiological settings and affecting prominently the world's most impoverished populations (Costa et al., 2015). Leptospirosis causes life-threatening manifestations such as pulmonary hemorrhage syndrome (LPHS) and Weil's disease and has emerged as a major worldwide cause of hemorrhagic fever and acute kidney injury (Ko et al., 1999).

Leptospira are highly motile and able to penetrate abraded skin and mucous membranes and cross tissue barriers, which facilitates hematogenous dissemination and systemic infection (Wunder et al., 2016). Infection in susceptible hosts manifests as an acute disease, however, a broad range of mammalian reservoirs can chronically carry the bacteria in their proximal kidney tubules. The bacteria are excreted through the urine and their ability to persist for weeks to months in the environment facilitates the transmission to humans, accidental hosts, coming into contact with *Leptospira* contaminated water or soil (Ko et al., 2009; Picardeau, 2017).

With more than one million cases each year, leptospirosis is a leading zoonotic cause of morbidity and mortality worldwide (Costa et al., 2015). Mortality from severe leptospirosis is high (>10%) despite aggressive supportive care. Diagnosis requires laboratory testing since the clinical presentation of early phase leptospirosis is non-specific and the epidemiological occurrences are foreign to the medical community. Current diagnosis is antiquated which hampers outpatient-based interventions aimed at reducing high mortality and contributes to the under-recognition of the disease (Riediger et al., 2017; Nabity et al., 2018). Limited understanding of leptospirosis determinants has hampered development of new diagnostic and therapeutic approaches. It is therefore essential to focus research on elucidating key virulence factors and pathogenesis mechanisms which will ultimately provide the knowledge needed for better diagnosis and treatment of leptospirosis. This compilation of research and review articles advances our understanding of the features and mechanisms that pathogenic leptospires adopt and explore to successfully establish infection in the host.

Adhikarla et al. described a novel-signaling pathway and the first virulence-associated two-component system (TCS) called *Leptospira* virulence regulator (lvr). This unique *Leptospira* TCS system, controls virulence and motility in pathogenic *Leptospira* and its characterization unveils the existence of a complex signaling network in this genus. Zhukova et al. provided the first genome-wide Transcriptional Start Site (TSS) and promoter maps for the pathogen *L. interrogans*. The authors analyzed the RNA from bacteria cultured under two temperatures, 30° and 37°C, revealing a major conservation of primary TSS at both temperatures. Furthermore, over 500 putative small regulatory RNAs (sRNAs) were identified, with regulatory functions yet to be characterized in this pathogen. These articles provide a framework for understanding how *Leptospira* can reprogram and adapt to the host, and contribute essential information for genetic studies.

OPEN ACCESS

Edited by:
John S. Gunn,
The Ohio State University,
United States

Reviewed by:
Brian Ahmer,
The Ohio State University,
United States

***Correspondence:**
Elsio A. Wunder Jr.
elsio.wunder@yale.edu
Azad Eshghi
azad@proteincentre.com
Nadia Benaroudj
nadia.benaroudj@pasteur.fr

Specialty section:
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *30 July 2018*
Accepted: *23 August 2018*
Published: *11 September 2018*

Citation:
Wunder EA Jr, Eshghi A and Benaroudj N (2018) Editorial: Pathogenesis of Leptospira. Front. Cell. Infect. Microbiol. 8:322. doi: 10.3389/fcimb.2018.00322

da Silva et al. determined that leptospiral extracellular proteases display proteolytic activity against host proteoglycans and plasma proteins, most likely with the participation of metalloproteases. Furthermore, they were able to demonstrate that attenuated and saprophytic species did not display proteolytic activity, indicating that the ability to degrade host molecules correlates with *Leptospira* virulence. Protein secretion in *Leptospira* is a relatively unexplored area of research and this study provided evidence that extracellular proteins of *Leptospira* likely contribute to the pathogenic mechanisms required for infection.

Cagliero et al. scrutinize the cytokine response caused by leptospiral infection and the different ensuing outcomes in susceptible and resistant animals, and how this host response can be used for diagnosis or control of the disease. Similarly, de Castro et al. described the role of the Complement System murine C5, to actively kill leptospires and control their dissemination during the early phases of the disease. Both articles give us important insights about the role of the innate immune system as a first and potentially essential response to *Leptospira* infection.

To answer the fundamental question in regard to the equilibrium between pathogenic leptospires and reservoir hosts, Nally et al. used the dialysis membrane chamber (DMC) (Caimano et al., 2014) in the rat model of chronic infection to identify differentially expressed proteins and post-translational modifications that are associated with the mammalian host signals, including 20 and 7 protein isoforms of LipL32 and LipL41, respectively. Furthermore, Nally et al. developed an inbred immunocompetent rat model to study the pathophysiological pathways involved in the chronic infection. Their results indicate that other than systemic immune response, the local lymphoid organ should be considered when studying renal colonization.

Santos et al. analyzed the whole-genome of 67 strains of *L. interrogans* serogroup Icterohaemorrhagiae, 55 and 12 isolates of serovars Copenhageni and Icterohaemorrhagiae, respectively. Serovars Copenhageni and Icterohaemorrhagiae are recognized to be the most virulent ones among all pathogenic species of *Leptospira*, and responsible for the majority of the reported severe cases. Their results showed that both serovars are closely related but with a distinct spatial clustering. Furthermore, they were able to identify a single indel as the only sequence variation between them with a high discriminatory power to genetically distinguish those two serovars.

Recent breakthroughs in genetic manipulation of *Leptospira* and whole-genome sequencing have provided tools and information to conduct research at the molecular level and better understand the biology and virulence of this unique spirochete. The collection of articles compiled in this research topic is exemplary in advancing our limited understanding of *Leptospira* pathogenesis and this information will support the development of new therapeutic and prevention approaches against this important yet neglected zoonotic disease.

AUTHOR CONTRIBUTIONS

EW drafted the editorial. NB, AE, and EW contributed to the writing and review process.

REFERENCES

Caimano, M. J., Sivasankaran, S. K., Allard, A., Hurley, D., Hokamp, K., Grassmann, A. A., et al. (2014). A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar Copenhageni. *PLoS Pathog* 10:e1004004. doi: 10.1371/journal.ppat.1004004

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015). Global morbidity and mortality of Leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

Ko, A. I., Galvao Reis, M., Ribeiro Dourado, C. M., Johnson, W. D. Jr., and Riley, L. W. (1999). Urban epidemic of severe leptospirosis in Brazil. Salvador leptospirosis study group. *Lancet* 354, 820–825. doi: 10.1016/S0140-6736(99)80012-9

Ko, A. I., Goarant, C., and Picardeau, M. (2009). *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* 7, 736–747. doi: 10.1038/nrmicro2208

Nabity, S. A., Hagan, J. E., Araujo, G., Damiao, A. O., Cruz, J. S., Nery, N., et al. (2018). Prospective evaluation of accuracy and clinical utility of the Dual Path Platform (DPP) assay for the point-of-care diagnosis of leptospirosis in hospitalized patients. *PLoS Negl. Trop. Dis.* 12:e0006285. doi: 10.1371/journal.pntd.0006285

Picardeau, M. (2017). Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat. Rev. Microbiol.* 15, 297–307. doi: 10.1038/nrmicro.2017.5

Riediger, I. N., Stoddard, R. A., Ribeiro, G. S., Nakatani, S. M., Moreira, S. D. R., Skraba, I., et al. (2017). Rapid, actionable diagnosis of urban epidemic leptospirosis using a pathogenic *Leptospira* lipL32-based real-time PCR assay. *PLoS Negl. Trop. Dis.* 11:e0005940. doi: 10.1371/journal.pntd.0005940

Thibeaux, R., Girault, D., Bierque, E., Soupe-Gilbert, M. E., Rettinger, A., Douyere, A., et al. (2018). Biodiversity of environmental *Leptospira*: improving identification and revisiting the diagnosis. *Front. Microbiol.* 9:816. doi: 10.3389/fmicb.2018.00816

Wunder, E. A. Jr., Figueira, C. P., Santos, G. R., Lourdault, K., Matthias, M. A., Vinetz, J. M., et al. (2016). Real-Time PCR reveals rapid dissemination of *Leptospira interrogans* after intraperitoneal and conjunctival inoculation of hamsters. *Infect. Immun.* 84, 2105–2115. doi: 10.1128/IAI.00094-16

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer BA and handling Editor declared their shared affiliation.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 23 February 2018
doi: 10.3389/fcimb.2018.00045

Lvr, a Signaling System That Controls Global Gene Regulation and Virulence in Pathogenic *Leptospira*

Haritha Adhikarla[1], Elsio A. Wunder Jr.[1], Ariel E. Mechaly[2], Sameet Mehta[3], Zheng Wang[4], Luciane Santos[1,5], Vimla Bisht[1], Peter Diggle[6], Gerald Murray[7], Ben Adler[7,8], Francesc Lopez[3], Jeffrey P. Townsend[4], Eduardo Groisman[9], Mathieu Picardeau[10], Alejandro Buschiazzo[2,11] and Albert I. Ko[1,5]**

[1] Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, United States, [2] Laboratory of Molecular & Structural Microbiology, Institut Pasteur de Montevideo, Montevideo, Uruguay, [3] Yale Centre for Genome Analysis, West Haven, CT, United States, [4] Department of Biostatistics, Yale School of Public Health, New Haven, CT, United States, [5] Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador, Brazil, [6] Lancaster Medical School, Lancaster, United Kingdom, [7] Department of Microbiology, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia, [8] Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, VIC, Australia, [9] Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT, United States, [10] Institut Pasteur, "Biology of Spirochetes" Unit, Paris, France, [11] Department of Microbiology, Institut Pasteur, Paris, France

OPEN ACCESS

Edited by:
Kathleen McDonough, Wadsworth Center, United States

Reviewed by:
Janakiram Seshu, University of Texas at San Antonio, United States
Ulisses Padua Pereira, Universidade Estadual de Londrina, Brazil

***Correspondence:**
Alejandro Buschiazzo
alebus@pasteur.edu.uy
Albert I. Ko
albert.ko@yale.edu

Received: 28 November 2017
Accepted: 08 February 2018
Published: 23 February 2018

Citation:
Adhikarla H, Wunder EA Jr, Mechaly AE, Mehta S, Wang Z, Santos L, Bisht V, Diggle P, Murray G, Adler B, Lopez F, Townsend JP, Groisman E, Picardeau M, Buschiazzo A and Ko AI (2018) Lvr, a Signaling System That Controls Global Gene Regulation and Virulence in Pathogenic Leptospira. Front. Cell. Infect. Microbiol. 8:45. doi: 10.3389/fcimb.2018.00045

Leptospirosis is an emerging zoonotic disease with more than 1 million cases annually. Currently there is lack of evidence for signaling pathways involved during the infection process of *Leptospira*. In our comprehensive genomic analysis of 20 *Leptospira* spp. we identified seven pathogen-specific Two-Component System (TCS) proteins. Disruption of two these TCS genes in pathogenic *Leptospira* strain resulted in loss-of-virulence in a hamster model of leptospirosis. Corresponding genes *lvrA* and *lvrB (leptospira virulence regulator*) are juxtaposed in an operon and are predicted to encode a hybrid histidine kinase and a hybrid response regulator, respectively. Transcriptome analysis of *lvr* mutant strains with disruption of one (*lvrB*) or both genes (*lvrA/B*) revealed global transcriptional regulation of 850 differentially expressed genes. Phosphotransfer assays demonstrated that LvrA phosphorylates LvrB and predicted further signaling downstream to one or more DNA-binding response regulators, suggesting that it is a branched pathway. Phylogenetic analyses indicated that *lvrA* and *lvrB* evolved independently within different ecological lineages in *Leptospira* via gene duplication. This study uncovers a novel-signaling pathway that regulates virulence in pathogenic *Leptospira* (Lvr), providing a framework to understand the molecular bases of regulation in this life-threatening bacterium.

Keywords: Leptospira, pathogenic, branched signaling, two-component system, hybrid histidine kinase, hybrid response regulator, virulence, gene duplication

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic species of the genus *Leptospira*. Infection with this pathogen results in more than one million human cases a year with a fatality ratio frequently exceeding 10% (Bharti et al., 2003; Costa et al., 2015a). The life cycle of pathogenic *Leptospira* is complex, including asymptomatic reservoir and susceptible hosts (Ko et al., 2009).

Large spectrum of mammalian hosts including rodents (the Norway rat, *Rattus norvegicus* and/or the black rat, *Rattus rattus*), live stock, dogs, and horses harbor and shed the pathogenic leptospires from their renal tubules into the environment (Ko et al., 1999; Costa et al., 2015b). Transmission of *Leptospira* to humans is due to exposure of risk groups to animal reservoirs or contaminated environments (Reis et al., 2008; Costa et al., 2015b). Therefore, pathogenic *Leptospira* must adapt rapidly to the versatile ecological niches encountered during its lifecycle. The *Leptospira* genomes encode an array of serine-threonine protein kinases (STPKs), extra cytoplasmic function (ECF) sigma factors, and two-component signal transduction systems (TCSs), which might enable *Leptospira* to traverse the diverse environmental stimuli experienced during the infection (Nascimento et al., 2004; Fouts et al., 2016). However, TCSs in *Leptospira* have been implicated only in heme metabolism (Louvel et al., 2008; Morero et al., 2014) and chemotaxis (Lambert et al., 2015) but not in virulence regulation. Here we provide a singular example of a virulence governing, non-classical TCS in pathogenic *Leptospira*.

The *Leptospira* genomes encode a substantially higher number of TCS genes (>50) compared to other spirochetes such as *Borrelia spp.* (<10) and *Treponema spp.* (<20). A pan-genus genomic analysis of globally representative 20 *Leptospira* species revealed that a high percentage of TCS genes (60%) in *Leptospira* encode non-classical TCSs (Fouts et al., 2016) and most of them are designated hybrid response regulators (HRR). Non-classical TCSs do not comply with a linear phosphate flow from sensor histidine kinases (HKs) to cognate response regulators (RRs), as observed in classical TCSs (Mascher et al., 2006).

HRRs comprise an N-terminal domain harboring the ultra-conserved aspartate residue that receives the phosphoryl group from upstream TCS partners (denominated receiver domain or REC), followed by C-terminal modules typical of HKs within the same polypeptide (Wuichet et al., 2010). HRRs remain largely unexplored with very few reports in endo-symbiotic bacteria and plant-associated bacteria (Wojnowska et al., 2013; Kaczmarczyk et al., 2015).

In this study, we report a novel hybrid histidine kinase / hybrid response regulator pair LvrAB (***L****eptospira* **v**irulence **r**egulator), specific to pathogenic *Leptospira*. Our findings determine the global regulatory role of *lvr* genes with a special emphasis on their role in virulence. Moreover, our study suggests that LvrA/B operate through a branched signaling pathway, predicting that a specific downstream DNA-binding response regulator(s) functions as final effector(s).

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

Leptospira interrogans serovar Manilae strain L495 WT (WT) and mutant strains (lvrA/B, lvrA/B II, lvrB, lic13192, lic13087, lic11713) were cultured in Ellinghausen-McCullough-Johnson-Harris liquid medium (EMJH) (Johnson and Harris, 1967) supplemented with 1% rabbit serum (Sigma-Aldrich) at 30°C with shaking (100 rpm). Leptospires were enumerated by dark-field microscopy in a Petroff-Hausser chamber (Thermo Fisher Scientific, Waltham, MA, USA). When appropriate, spectinomycin or kanamycin was added to the culture medium at the final concentration of 40 μg/ml.

Motility of the WT and mutant Leptospira (lvrA/B, lvrA/B II, lvrB) was evaluated by inoculating 105 leptospires onto semisolid EMJH medium containing 0.5% agar (DifcoTM Noble Agar, BD Biosciences, NJ, USA). The plates were incubated at 30°C without shaking and the diameters of the growth zones were measured after 14 days. Assays were repeated in triplicate.

Random Mutagenesis

A plasmid vector pSC189 containing both the hyperactive transposase C9 and transposon terminal inverted repeats flanking a kanamycin resistance gene (Bourhy et al., 2005) was used to deliver Himar1 in the L. interrogans serovar Manilae strain L495 genome and random mutants were selected. Representative Kmr clones were further tested by PCR for the identification of the insertion site of the Himar1 transposon in the genome using primers flanking the site of insertion, followed by DNA Sanger sequencing (Table S2).

Site-Directed Mutagenesis of Lvr Proteins

LvrA and LvrB coding genes were amplified from *L. interrogans* serovar Copenhageni Fiocruz L1-130 genomic DNA, and cloned into a modified version of the pQE80L (Qiagen Kit, Germantown, MD, USA) plasmid using the Restriction Free (RF) method (Van Den Ent and Lowe, 2006). This plasmid (pQE80L-TEV) includes a TEV cleavage site and a GSGS linker after the N-terminal His-tag. RF cloning, using the appropriate mutagenic oligonucleotides also generated point mutants. Full-length LvrA or LvrB protein variants were expressed in *Escherichia coli* (TOP10F′) and purified by affinity chromatography using standard procedures.

Virulence Test

In vivo screening was performed to test the virulence potential of Leptospira WT and mutant strains (lvrA/B, lvrA/B II, lvrB, lic13192, lic13087, lic11713). Golden Syrian male hamsters were challenged via conjunctival route with doses of 5 × 106, 107, or 108 leptospires in 10 μl of EMJH, as described previously (Wunder et al., 2016). Animals were monitored for clinical signs of disease up to 21 days post-challenge. Sick animals were immediately euthanized by inhalation of CO2, and death was used as primary outcome. Treatment effects (mutations in L. interrogans Manilae strain) and day effects were estimated based on proportional hazards model.

For studying expression pattern of *lvr* genes *in vivo*, golden Syrian male hamsters ($n = 2$) were challenged with *Leptospira* WT (10^8 leptospires in 1 mL of EMJH) via intraperitoneal (IP) route as described previously (Wunder et al., 2016). Animals were monitored for clinical signs of disease up to 21 days post-challenge and sick animals were immediately euthanized by inhalation of CO_2. Blood samples were collected by retro-orbital bleeding procedure from these hamsters at defined intervals (1 day, 3 days, 5 days, and 7 days) and stored in TRIzol (Invitrogen^{TM}, Thermo Fisher Scientific, Waltham, MA, USA) at −80°C until further use.

Ethics Statement

All animal protocols and work were approved and conducted under the guidelines of the Yale Institutional Animal Care and Use Committee (IACUC), under approved protocol #2014–11424. The Yale IACUC strictly adheres to all Federal and State regulations, including the Animal Welfare Act, those specified by Public Health Service, and the US Department of Agriculture, and uses the *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* as a guide for all animal studies.

RNA Extraction, Library Preparation, and RNA-Sequencing

Leptospira cells of WT and mutant strains (*lvrA/B* and *lvrB*) were cultured in EMJH (Johnson and Harris, 1967) supplemented with 1% rabbit serum (Sigma-Aldrich, St. Louis, MO, USA) at 30°C with shaking (100 rpm) and subsequently harvested at late-log phase by centrifugation at 3,200 *g*. RNA was extracted for two biological replicates using the TRIzol method (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Six strand-specific sequencing libraries, two for each strain, were produced from total RNA. The libraries were run on HiSeq 2000, generating approximately between 21 and 35 million pair-end reads of 76 bp each. Adapter sequences, empty reads, and low-quality sequences were removed. The first and the last nucleotides with quality scores below 20 for each read were trimmed to remove low quality bases using in-house scripts. After trimming, reads shorter than 45 bp were also discarded. Trimmed reads were mapped to *L. interrogans* serovar Manilae L495 with a known transcriptome index (http://www.genoscope.cns.fr/) using Tophat v2.0.11 (Trapnell et al., 2009). Only reads that mapped to a single unique location in the genome with a maximum of two mismatches in the anchor region of the spliced alignment were reported in these results. To obtain a tally of the number of the reads that overlapped the exons of a gene, we analyzed the aligned reads with HTSeq v0.6.1p1 (Anders et al., 2015) (http://www-huber.embl.de/users/anders/HTSeq/doc/). Differential expression analysis was performed with DESeq2 (Love et al., 2014). *P*-values were corrected for multiple testing with Benjamini-Hochberg correction.

We identified 850 genes with at least 2 $\log_2$-fold changes and with a significance of p-adjusted < 0.05 in each comparison group. Hierarchical clustering was performed with 850 genes exhibiting significant changes across all conditions and a heat map was generated with the regularized-logarithm transformation of the data (Schmittgen and Livak, 2008). PCA plots showed samples clustering by treatment group.

RNA Seq Data Analyses

The sequences were aligned to the *L. interrogans* serovar Manilae L495 obtained from http://www.genoscope.cns.fr. The reference genome was indexed using the bowtie2-index. The reads were pre-processed to remove the first and last base, and filtered for quality using scripts written in PERL. The reads were aligned to the reference genome using Tophat (Trapnell et al., 2009). The aligned bam files were processed using htseq-count (Anders et al., 2015), and the raw counts were processed using DESeq2 package in R (Love et al., 2014). The downstream analysis and plotting was done using home brew scripts written in R (R Archive Network). R package pheatmap was employed for the heatmap where the values were scaled according to row to depict how the gene expression for the specific gene changed across the conditions.

Whole Genome Sequencing

L. interrogans serovar Manilae mutant strains (*lvrA/B* and *lvrB)* were cultured in Ellinghausen-McCullough-Johnson-Harris liquid medium (EMJH) (Johnson and Harris, 1967) supplemented with 1% rabbit serum (Sigma-Aldrich, St. Louis, MO, USA) at 30°C with shaking (100 rpm). DNA was then extracted from late-log phase cultures by Maxwell 16 DNA purification kit (Promega, Fitchburg, WI, USA). The quality and concentration of DNA was measured by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by fluorometric assay using the Quanti-iT PicoGreen dsDNA assay kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

The genomes of the isolates *lvrA/B* and *lvrB* were sequenced at the Yale Center for Genome Analysis (YCGA) using the Illumina HiSeq2000 sequencing system. The sequenced reads were mapped to *L. interrogans* serovar Manilae L495 genome (http://www.genoscope.cns.fr/) and Geneious software package was employed for variant calling. In order to confirm a variant call, a minimum of 75% of the sequencing reads should support the call.

Quantitative Reverse Transcription PCR (RT-qPCR) for Target Gene Identification and RNA Seq Data Validation

Leptospira WT and *lvr* mutants were cultured till mid-log and late-log phases. RNA was extracted for two biological replicates using the TRIzol method (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). For studying expression of *lvr* genes *in vivo*, total RNA was extracted from hamster blood samples ($n = 2$) stored in TRIzol, as per the manufacturer's instructions (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Ambion® TURBO DNA-free™ DNase treatment kit (Applied Biosystems Inc, Foster City, CA, USA) was employed to remove contaminating DNA from RNA preparations. The concentration of RNA was determined using a NanoDrop™ 2000 spectrophotometer system (Thermo Fisher Scientific, Waltham, MA, USA). The High capacity cDNA reverse transcription kit (Applied Biosystems Inc, Foster City, CA, USA) was employed for conversion of total RNA to single-stranded cDNA. The primers designed for target gene identification and RNA-seq data validation are listed in Table S2.

The qPCR was carried out on 7500 fast real-time PCR (Applied Biosystems Inc, Foster City, CA, USA) using iQ™ SYBR® Green supermix (Bio-rad, Hercules, CA, USA) according to manufacturer's instructions. The thermal cycling conditions used in the qPCR were 95°C for 3 min, followed by 40 cycles

of 95°C for 5 s and 60°C for 1 min. The specificity of the SYBR green PCR signal was confirmed by melt curve analysis. In RT-qPCR experiments, *flaA* gene (flagellar apparatus gene) was used as an endogenous control and *L. interrogans* WT was employed as reference strain. A relative quantification analysis was performed using the comparative CT method, and the relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Molecular Evolution Analyses

Multiple BLAST searches were performed against the NCBI database for homologs of *Lvr* in other bacterial genomes. Two Lvr homologs in *Mycobacterium* were employed to root the phylogeny. A set of 67 unique amino acid sequences was assembled and they were aligned using SATé-II (Liu et al., 2012) with MAFFT as aligner, MUSCLE as merger, and RAxML as tree estimator under WAG model. The robustness of branching topologies was estimated with 1,000 maximum likelihood searches of bootstrapped sequence data using PhyML (Guindon et al., 2010) under the WAG model. Robustness of the topology was further confirmed with Bayesian analyses using MrBayes 3.2 (Ronquist et al., 2012). Bayesian phylogenetic analyses were performed using the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC) under a mixed amino acid model by running for chains with 10,000,000 generations. Trees were sampled every 1000th generation, and first 2,000 trees were discarded before computing a consensus tree. Branches with bootstrap proportions (BP) higher than 80% or posterior probability (PP) higher than 0.98 were considered significantly supported.

A total of 67 sequences with 1,199 aligned positions were included in the phylogenetic analyses. The tree was rooted with orthologs of *Mycobacterium* (*M. gastri* and *M. kansasii* genes, with 42% identity). There were no significant conflicts in topology of the gene trees as inferred by maximum likelihood analyses using PhyML and Bayesian analyses; branch support was significant (bootstrap >80% and posterior probabilities ≥0.98). A consensus tree based on the last 8,001 trees sampled per 1,000 generations in Bayesian analyses featured a Bayesian posterior probability of ≥98%.

Phosphotransfer Assays

Purified Lvr proteins (1 mg/mL) in desalting buffer (25 mM Tris-HCl, 250 mM NaCl, 10 mM MgCl2, pH 8.5) were incubated with 1 mM ATP and 10 mCi of [γ^{33}P] ATP (3000 Ci/mmol, ARC) at room temperature for 30 mins. Phosphotransfer reactions were stopped by mixing the samples with Laemmli buffer. Samples were then subjected to gel electrophoresis (SDS-PAGE), and visualized by autoradiography (Hyperfilm ECL) after 1–3 days of exposure. Densitometry analysis was performed using the software ImageJ.

Statistical Analysis

GraphPad Prism (Prism Mac 5.0) was employed for statistical analysis of *in vivo* and motility data. Fisher's exact test was used to calculate *p*-values for difference in mutant infections clearance with 2 × 2 contingency table. The Benjamini-Hochberg FDR method was used to adjust the *p*-values for multiple testing of RNA-seq data (Noble, 2009). Spearman and Pearson's *R*-value determined similarities between RNA replicates.

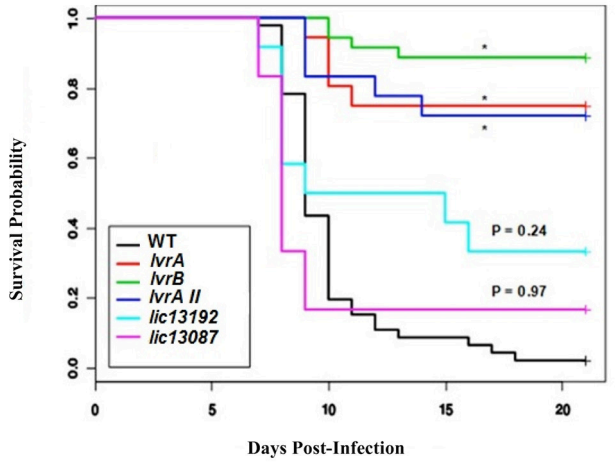


FIGURE 1 | *In vivo* screening of selected *Leptospira* TCS mutants. Golden Syrian male hamsters were challenged with wild type, *lvrA/B* (M1529), *lvrA/BII* (M1529 II), *lvrB* (M1419), *lic13192* (M480), and *lic13087* (M854) mutants of *L. interrogans* Manilae L495 sp via conjunctival route in doses of 5×10^6, 10^7, and 10^8 leptospires. Animals were monitored for 21 days post-challenge with death as a primary outcome. The survival probability plot was based on a proportional hazards model. Treatment effects (mutations in *L. interrogans* Manilae strain) and day effects were estimated based on this model and *P* values were calculated. *$P < 0.0001$.

Data Availability

RNA-Seq reads are available in the NCBI Sequence Read Archive, under accession numbers *L. interrogans* Manilae wild type (I: GSM2085874, II: GSM2085875), *lvrA/B* mutant (I: GSM2085878, II: GSM2085879), *lvrB* mutant (I: GSM2085876, II: GSM2085877).

The genome sequencing data for *lvrA/B* and *lvrB* mutants have been deposited in NCBI under accession numbers SRR5956150 and SRR5956154 respectively.

RESULTS AND DISCUSSION

Identification of a TCS Governing Virulence in *Leptospira*

Our comparative genomic analysis of 20 *Leptospira* species (Fouts et al., 2016) led us to the identification of seven genes encoding TCS proteins in all the pathogenic *Leptospira* species but distinctly absent from saprophytic spp. Therefore, we speculated the role of these seven signaling proteins in pathogenic mechanisms and/or virulence modulation. As a further step to identify the regulatory role of these putative signaling proteins, we screened our *Mariner* transposon based mutant library in pathogenic *L. interrogans* serovar Manilae. In our screening of mutant library, we identified disruptions in four pathogen-specific TCS genes (Tables S1, S2). Two of the mutants had insertions in a pathogen-conserved locus encoding a hybrid HK

(gene *lic11709*) and a hybrid RR (gene *lic11708*). The other two mutant strains were found to have insertions in a classical HK gene (*lic13087)* and a hybrid HK gene (*lic13192*) (Table S1).

Subsequently, we aimed to determine the ability of the above TCS mutants to cause infection in a hamster model. A significant loss of virulence ($p < 0.05$) was observed in the survival curves of hamsters infected with *lic11708* and *lic11709* mutants when compared to wild-type *L. interrogans* (WT) (**Figure 1**, Tables S3, S4). By contrast, *lic13192* and *lic13087* mutants did not show significant decrease in hamster mortality ($p > 0.05$; **Figure 1**). All four strains showed similar bacterial growth in EMJH medium (data not shown) indicative that virulence defects of the *lic11708* and *lic11709* mutants are due to their altered virulence potential in a mammalian host. Taking into account the *in vivo* virulence attenuation phenotype, this TCS pair is designated as Lvr, ***L****eptospira* **v**irulence **r**egulator system, comprising proteins LvrA (*lic11709*) and LvrB (*lic11708*).

Whole genome sequencing of *lvrA* (*lic11709*) and *lvrB* (*lic11708*) mutants revealed single transposon insertion events in corresponding *lvr* genes respectively. Thus, we confirmed that the attenuated phenotype exhibited by these mutant strains is attributable to specific insertions in *lvr* genes but not due to any off-target mutations. Genome sequencing results therefore compensated for our unsuccessful attempts to complement the *lvr* mutant strains with corresponding wild-type genes. Furthermore, an independent transposon mutant *lvrA*_II was additionally identified in *lvrA* gene locus (Table S1) and it also displayed an *in vivo* attenuated phenotype (**Figure 1**). Taken together, the evidence provided here strongly supports a virulence modulation function of the Lvr signaling system in pathogenic *Leptospira* spp.

Lvr Constitutes an Unusual "Hybrid Histidine Kinase/Hybrid Response Regulator" Pair

Pathogenic *Leptospira* spp. uniquely harbor the genomic region encompassing two genes, *lvrA* and *lvrB,* which appear to be part of the same operon (**Figure 2A**). Consistent with this notion, the *lvrA* and *lvrB* transcripts were 29- and 9- fold lower than the wild type strain in an *lvr* mutant with a transposon insertion in the *lvrA* gene (**Figure 2B**). Hereafter, *lvrA* strain is thus named as *lvrA/B* mutant. By contrast, the *lvrB* mutant with a transposon insertion in the intergenic region between *lvrB* and *lvrA* resulted in a 30-fold downregulation of the *lvrB* transcript only, with *lvrA* expression unchanged (**Figure 2B**).

The *lvr* genes encode for TCS proteins with unusual domain architectures (Goulian, 2010). The 832 amino acid long LvrA is a hybrid HK (HHK) protein, with a canonical N-terminal HK region and a C-terminal RR region within the same polypeptide (**Figure 2C**). The HK region comprises a PAS sensory domain (spanning residues 11–141), a Dimerization and Histidine phosphotransfer (DHp) domain (206–270) and a Catalytic and ATP-binding (CA) domain (residues 325–427). The RR region that follows is unusual in that it comprises a typical REC domain (residues 478–583) that is followed by an effector domain resembling a HK on its own (**Figure 2B**), comprising a DHp domain (residues 604–670) and CA domain (residues 720–823). Each of the two HK portions in LvrA includes the histidine residue within the conserved H-box motifs, which could thus be phosphorylated during signaling, at positions 218 and 616. A potentially phosphorylatable aspartate residue within LvrA's RR domain is found at position 524 (**Figure 2B**).

The 382 amino acid long LvrB protein has a domain organization corresponding to a HRR, similar to the C-terminal half of LvrA (**Figure 2C**). LvrB thus comprises a REC domain (residues 7–120), including conserved Asp56 at the putative phosphorylation site, upstream of an HK core. The latter displays a DHp domain (residues 149–213) harboring a phosphorylatable His at position 161, and a CA domain (residues 264–374; **Figure 2C**). By using an *in silico* approach [http://topcons.cbr.su.se/] we predicted the cytoplasmic localization of Lvr proteins. Also absence of transmembrane regions in LvrA or LvrB proteins confirms their cytoplasmic localization. Interestingly, DNA binding domains are equally absent in both Lvr proteins, instead their corresponding REC domains are connected to enzymatic (HK) effector domains.

We hypothesized that the LvrA and LvrB proteins are cognate partners within a phosphotransfer cascade pathway, because the *lvrA* and *lvrB* genes are co-transcribed as part of an operon. We tested this hypothesis by quantifying phosphotransferase activity *in vitro* using wild type (LvrB_wt) and mutant versions of full-length LvrA and LvrB proteins, purified as soluble recombinant species from *E. coli*. We employed autokinase-defective mutant LvrB_H161 as a negative control in which the phosphorylatable His161 was substituted by alanine (LvrB_H161A). Incubation of LvrB_H161A with variants of LvrA (wild-type, LvrA_H218A, and LvrA_D524A) in the presence of [γ^{33}P] ATP, showed that LvrA catalyzes the transfer of its His-bonded phosphoryl group to LvrB (**Figure 2D**). To study the effect of Asp56 phosphorylation on the autokinase activity of LvrB, we performed autophosphorylation assays in the presence or absence of LvrA_D524A (**Figure 2E**). The autokinase activity of wild-type LvrB significantly increased in presence of its partner, respect to the basal level of activity detected in its absence. In contrast, the activity of the LvrB_D56A mutant was not affected, displaying basal activity levels in both cases. Taken together, these results suggest that phosphotransfer occurs from LvrA His218 to the receiver Asp56 within LvrB, indeed supporting the hypothesis that LvrA and LvrB are part of the same signaling pathway.

LvrAB Modulates Global Transcriptional Regulation

To uncover the physiologic role of the Lvr system, we examined the global gene expression pattern of the *lvrA/B* and *lvrB* mutant strains by comparing their transcriptome profile to that of the WT *Leptospira*, all grown under standard *in vitro* conditions (30°C in EMJH). Principal Component Analysis (PCA) showed no significant variations between replicates of these RNA seq data (Figure S1). Differential gene expression analyses revealed significant changes in the transcription of 324 genes (∼7.5% of the genome) in the *lvrA/B* mutant, 212 genes (∼4.9%) in the *lvrB* mutant, and 314 genes (∼7.2%) in the *lvrA/B* n *lvrB* set

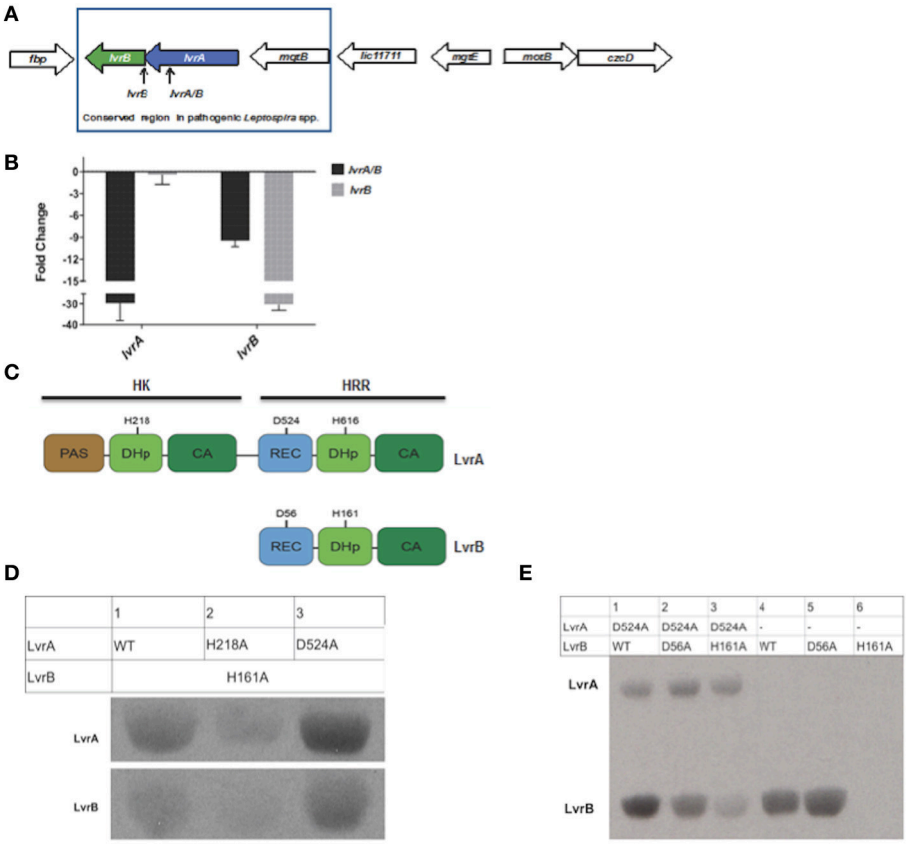


FIGURE 2 | Gene arrangement and domain organization of Lvr dyad in pathogenic *Leptospira* spp. **(A)** This figure is a schematic representation of the Lvr loci in pathogenic Leptospira. Arrow length is proportional to the gene length. *lvrA* (blue): Putative hybrid histidine kinase-response regulator gene and *lvrB* (green): Putative hybrid response regulator gene in *L. interrogans* serovar Manilae strain L495. Himar1 insertion site in *lvrA* gene (*lvrA/B* mutant) and in intergenic region between *lvrA* and *lvrB* (*lvrB* mutant) has been indicated. **(B)** *In vitro* gene expression of *lvrA* and *lvrB* by RT-qPCR **(C)** Domain organization of Lvr proteins; signature segments of kinase and receiver domains were identified by multiple sequence alignment and indicated. Conserved histidine and aspartate residues as putative phosphorylation sites are denoted for each protein. REC, receiver domain; PAS, Per, Arnt, Sim domains; DhP, Dimerization histidine phosphotransfer; CA, catalytic and ATP-binding domain. **(D)** Phosphotransfer from LvrA to LvrB. An LvrB mutant devoid of autokinase activity (LvrB_H161A) was incubated with [Y-33P] ATP for 30 min with LvrA wild type, LvrA_H218A or LvrA_D524A (as labeled in each lane). Reaction products were separated by SDS-PAGE and visualized using autoradiography. **(E)** Wild-type LvrB and the point mutants D56A and H161A were incubated with [Y-33P] ATP and MgCl2 for 30 min, in presence (lanes 1 to 3) or absence (lanes 4 to 6) of LvrA_D524A (as labeled in each lane). Reaction products were separated by SDS-PAGE and visualized using autoradiography.

(**Figure 3A**). Remarkably, 540 genes were derepressed in both *lvr* mutant strains, in comparison to 310 downregulated genes. To validate these gene expression data, we performed quantitative RT-PCR (qRT-PCR) for 17 randomly selected genes from the panel of differentially expressed genes (log $\geq$ 2-fold; adjusted p-value ≤ 0.05). There was a strong agreement between our RNA-Seq and qRT-PCR datasets with a correlation coefficient (R^2) of 0.9119 across the entire panel (**Figure 3B**).

We employed hierarchical clustering of *lvr* gene expression profiles to uncover biologically relevant expression signatures (**Figure 3C**). The effect of *lvr* gene inactivation on transcript levels indicated a positive role for Lvr in clusters 1, 4, and 5, and a negative role in clusters 2 and 3 (**Figure 3D**). To evaluate the potential biological role of the differentially expressed genes we assessed the cluster of orthologous genes (COG) classification system (**Figure 3E**, Tables S5A–F). This analysis identified the distribution of differentially expressed genes in all 20 COG categories (**Figure 3E**, Table S6). We observed a significantly increased expression of genes involved in translation (27.6%) and energy production (15.2%), which were distributed in clusters 1 and 5 (**Figure 3D**). Interestingly, disruption of the Lvr dyad affected 11 distinct operons (47 genes) corresponding to the protein translation machinery (Table S7). This pattern resembles the complex regulation pattern of a cyanobacterial global regulator, PipX, involving 16 genes linked to translation (Espinosa et al., 2014). Additionally, there was an increased expression of genes belonging to signaling and regulation categories (transduction, transcription), primary metabolic processes (lipid transport, nucleotide transport) and metabolic adaptation (cell motility, post-translational modification, and defense mechanisms) in clusters 1, 4, and 5. Genes of unknown function were distributed among all five clusters with relative abundance in downregulated clusters 2 (42.6%) and 3 (57.5%) (**Figures 3D,E**).

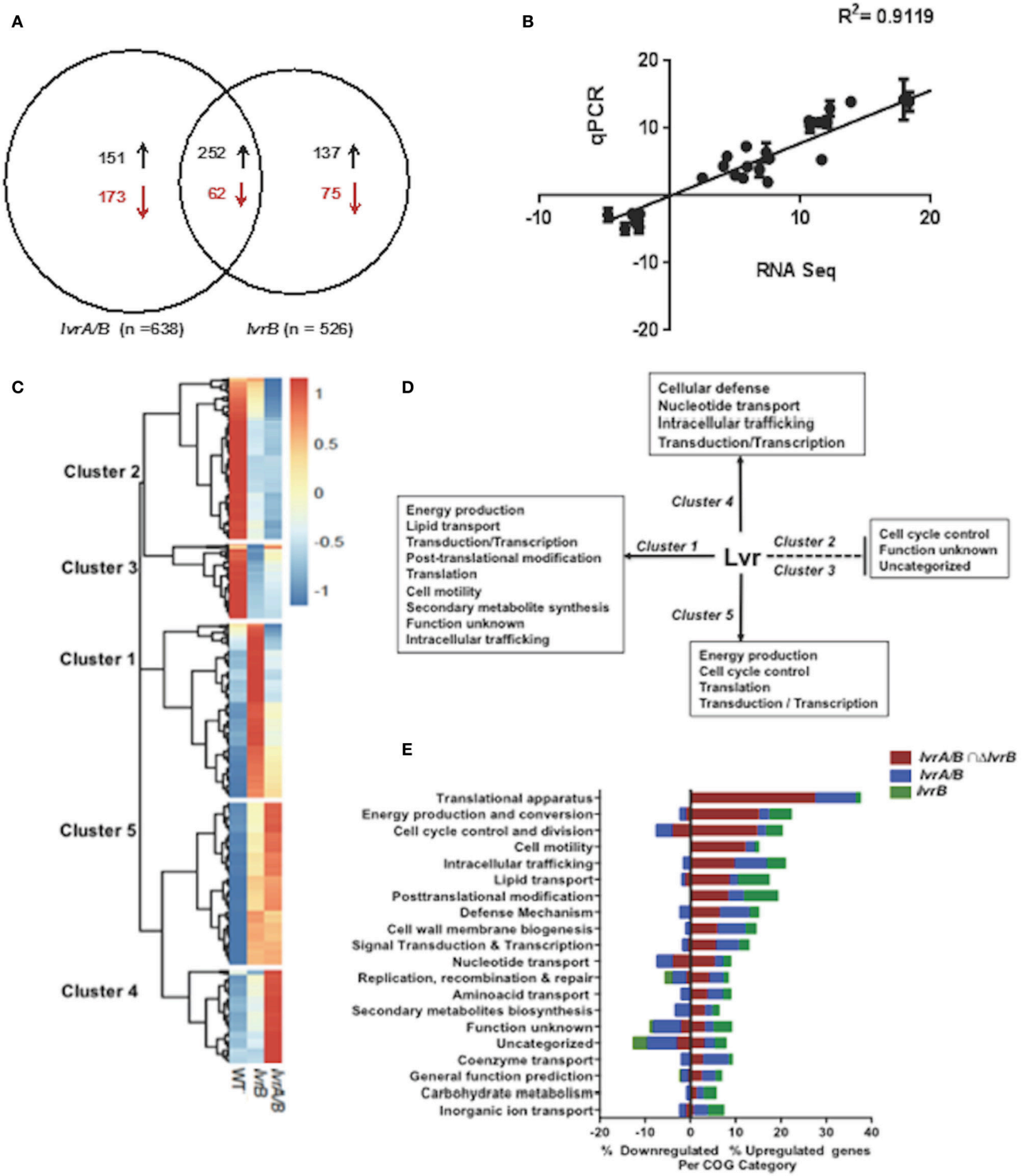


FIGURE 3 | Global transcriptional changes in *lvr* mutants. **(A)** Venn diagram depicting the number of differentially expressed genes in *lvr* mutants, *lvrA/B* (M1529) and *lvrB* (M1419) with ± log 2-fold change cut-off and $P \leq 0.05$. **(B)** Validation of RNA-Seq Analysis was performed by RT-qPCR and correlation coefficient has been indicated. **(C)** Heatmap depicting clusters of differentially expressed genes in *lvr* mutants when compared to *L. interrogans* Manilae L495 WT. Computationally we identified five arbitrary clusters that are marked in the heat map. **(D)** Lvr regulatory functions inferred from transcriptome analysis of *lvr* mutants, *lvrA/B* (M1529) and *lvrB* (M1419). Solid and dashed lines depict positive regulation and negative regulation, respectively. Inferences are based on relative abundance of COG categories (>5%) across each cluster. **(E)** Functional categorization of upregulated and downregulated genes in *lvr* mutants, *lvrA/B* (M1529) and *lvrB* (M1419) during late-log phase of growth at 30°C. Percent distribution is calculated for the total number of differentially expressed genes (according to the RNA-Seq analysis; log 2-fold change, $P < 0.05$) in each COG category.

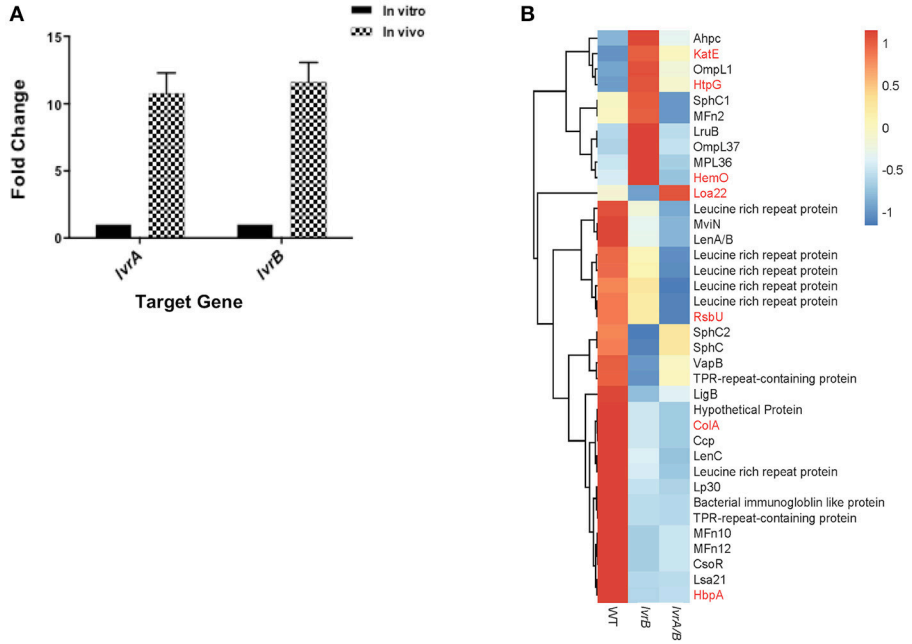


FIGURE 4 | Lvr dyad governs leptospiral virulence. **(A)** *In vivo* expression of *lvr* genes determined by RT-qPCR. Relative expression of the target *lvr* genes was studied by quantifying transcripts in sample collected from blood of hamsters ($n = 2$) at 3 days post-infection intraperitoneally with *L. interrogans* serovar Manilae WT at a dose of 10^8 *leptospires*. Transcripts of *in vitro* cultures were obtained from a late-log phase culture of *L. interrogans* serovar Manilae WT incubated in EMJH at 30°C. *In vivo* results represent the expression levels of *lvr* genes in comparison to *in vitro* conditions and normalized to *flaB* gene expression. Results are the average of two independent assays and the error bars indicate ±1 SD. **(B)** Categorization of differentially expressed virulence-related genes ($P < 0.05$) in *lvrA/B* (M1529) and *lvrB* (M1419) mutants into genetically characterized (red) genes and putative (black) genes.

Taken together, we discovered by global expression analysis that Lvr signaling system modifies the expression of ∼15% chromosomal genes in *Leptospira* (**Figure 3E**). This global regulatory role is reminiscent of the *Streptococcus pyogenes* TCS CsrRS (CovRS), which influences the transcription of 15% of its chromosomal genes (Graham et al., 2002).

LvrAB Governs Virulence in *Leptospira*

Virulence attenuation of *lvr* mutants allowed us to hypothesize that the Lvr system regulates virulence genes in *Leptospira*. *In vivo* assessment of *lvr* gene expression indicated that both *lvrA* and *lvrB* genes were upregulated (9-fold and 12-fold, respectively) in hamster blood, 3 days post-infection via intraperitoneal route with a lethal dose of *Leptospira* WT (**Figure 4A**). This data is consistent with the direct role of the Lvr system in the infectious process of *Leptospira*.

Furthermore, in our global expression analyses proven and putative virulence genes were differentially regulated in *lvr* mutants. *Leptospira* genes encoding for characterized virulence factors such as collagenase (*colA*), serine phosphatase (*rsbU*) and *h*emin-*b*inding *p*rotein A (*hbpA*) (Asuthkar et al., 2007; Eshghi et al., 2014; Kassegne et al., 2014), were repressed in the *lvr* mutants compared to the wild type strain (**Figure 4B**). Genes encoding for extracellular matrix proteins with a proposed role in pathogen-host interactions (*ompL1*, *mfn2*, *lruB, mpl36*, *lenA/B*, *lenC*, *lp30, mfn10, mfn12*, *lsa21*; Pinne et al., 2012; Vieira et al., 2014) and for those implicated in host adaptation (*sphC*, *sphC1*, *sphC2*, *mviN*; Caimano et al., 2014) were repressed in *lvr* mutants (**Figure 4B**). Putative virulence-related genes were also found to be repressed particularly those encoding leucine rich repeats (*lrr*) (Miras et al., 2015), tetratricopeptide repeats (*tpr*) (Cerveny et al., 2013), bacterial immunoglobulin (*mviN*) (Caimano et al., 2014), and copper homeostasis protein (*csoR*) (Liu et al., 2007) (**Figure 4B**). Taken together, these results indicate that the Lvr system has a major role in mediating virulence regulation through a complex network of genes that ultimately affect the pathogenic potential of *Leptospira*.

Lvr Regulates Cell Motility

Interestingly, there was an increased expression of cell motility related genes in both *lvrA/B* (10.37%) and *lvrB* (12.26%) mutants. These genes include those with predicted roles in chemotaxis, as well as flagellar apparatus-related transcripts (**Figure 5A**). Upon testing of the motility pattern in semisolid media, the *lvrA/B* mutant exhibited an increased spreading phenotype (32.5 mm ± 1) compared to the wild-type strain (20.5 mm ± 1). However, the motility pattern exhibited by the *lvrB* mutant (21 mm ± 0.77) resembled that of the wild-type *Leptospira* (**Figures 5B,C**). That the *lvrA/B* mutant displayed a distinct phenotype, and not the *lvrB* mutant, supports the most likely hypothesis that LvrA can operate independent of LvrB.

Taken together, the Lvr signaling system seems important to reprogram leptospiral motility. However, the regulation of motility by Lvr is intriguing and warrants further investigation

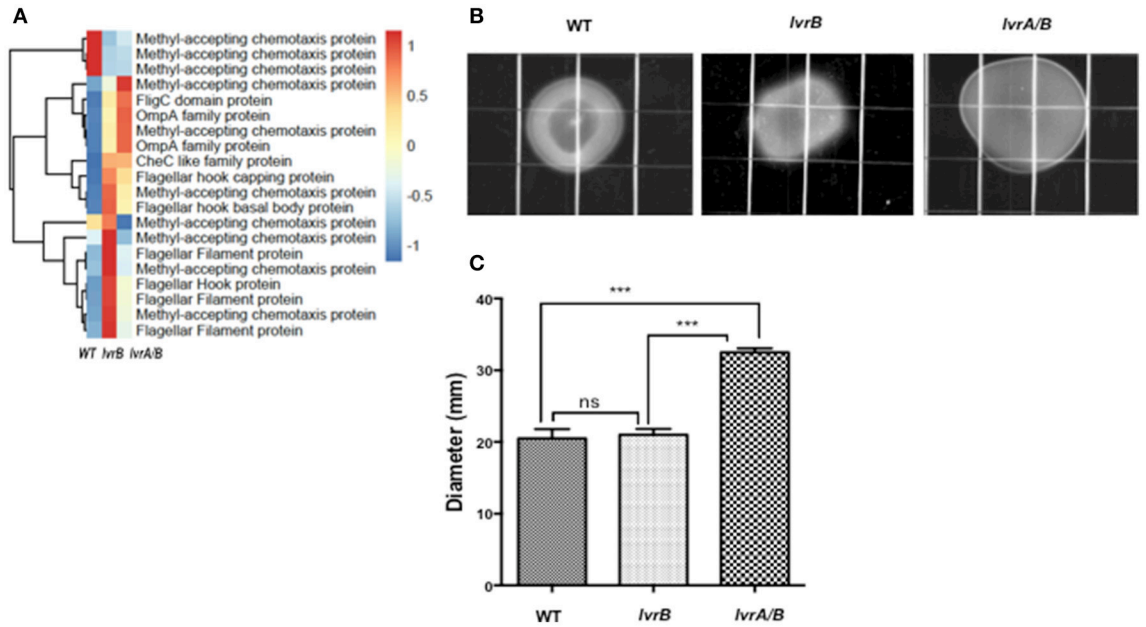


FIGURE 5 | Lvr dyad regulates motility of *Leptospira* spp. **(A)** Hierarchical clustering heatmap representing the normalized expression levels of indicated motility genes in *Leptospira* Manilae L495 wild type, *lvrA/B* (M1529) and *lvrB* (M1419) mutants. **(B)** EMJH plates (0.5% agar) inoculated with 10^5 cells of *Leptospira interrogans* Manilae L495 wild type strain or *lvrA/B* (M1529) and *lvrB* (M1419) mutants. Plates were incubated at 30°C and colony diameter was measured on 14th day. A representative plate from one of the three experiments is shown. Images were captured by Chemidoc XRS system (BioRad) **(C)** Graphical representation of colony diameter for *Leptospira interrogans* Manilae L495 wild type, *lvrA/B* (M1529) and *lvrB* (M1419) mutants measured after 17 days of incubation on 0.5% semisolid media. Points are plotted at the mean of three biological replicates and error bars indicate ±1 SD. ***$P \leq 0.001$; ns, not significant.

because of the complexity of the flagellar apparatus in *Leptospira* and the lack of understanding of its molecular mechanisms (Wunder et al., 2016).

Evolution of the LvrA/B System

The genomic region comprising the *lvrA* and *lvrB* genes was found only in pathogenic *Leptospira* species, suggesting that these genes could have been acquired through lateral gene transfer. To test this hypothesis, phylogenetic analyses were performed on an alignment of *lvr* gene orthologs in pathogenic *Leptospira* species (with >80% identity), also including putative hybrid HK/hybrid RR hits in intermediate species (with ∼50% identity) and saprophytic species (with <30% identity). PanOCT ortholog clustering identified the hits from intermediate *Leptospira* species as divergent paralogs with a different gene neighborhood than in pathogens (Fouts et al., 2016). Our search for *lvr* orthologs in non-*Leptospira* genomes revealed closest hits (with ∼40% identity) in gram-positive *Mycobacterium* spp., beta proteobacteria *Dechlorosoma* spp., and gamma proteobacteria *Legionella* spp., suggesting that these genes are of ancient origin. We identified six *lvr* orthologs in *Legionella* (with 40% identity and these orthologs clustered with *lvrA* of *Leptospira* (**Figure 6**). *Lvr* and its gene family are absent from other spirochetes such as *Treponema* and *Borrelia*. These results are consistent with the notion that *Leptospira* acquired a progenitor of *lvr* through lateral gene transfer.

Our phylogenetic analyses indicated that genes specifying the Lvr signaling system experienced at least one gene duplication event after the acquisition of the progenitor *lvr* genes (**Figure 6**). This is because distinct clades were observed for intermediate vs. pathogenic *Leptospira* species. Furthermore, PanOCT ortholog clustering of paralogs from intermediate species suggests lineage-specific duplication (**Figure 6**). For instance, search for a PAS domain-encoding *lvrA* did not yield any hits in the saprophytic species *L. biflexa*, suggesting a specialized role for this domain in expanding the signaling capabilities of pathogenic *Leptospira*. Likewise, putative RRs identified from saprophytic *Leptospira* species showed a distant evolutionary origin with *lvrB* orthologs of intermediate species, but not with pathogenic *Leptospira*. This study supported the proposition that duplicated genes relevant to virulence would become fixed in pathogenic lineages, while they could be lost in saprophytic lineages (Powell et al., 2008). Therefore, Lvr proteins constituting regulatory networks in pathogenic *Leptospira* spp. most likely provides a selective advantage related to their infectious ability and lifestyle diversity.

Conceptual Model for Lvr Regulatory System

Activation of the Lvr TCS might depend upon signal sensing by the single sensory PAS domain found in LvrA protein (**Figure 7**). The fact that *lvr* genes are induced *in vivo* after host infection (**Figure 4A**), strongly suggests that a specific input signal(s), yet to be determined, is present within the host environment. In cytoplasmic HKs, PAS domains may mediate

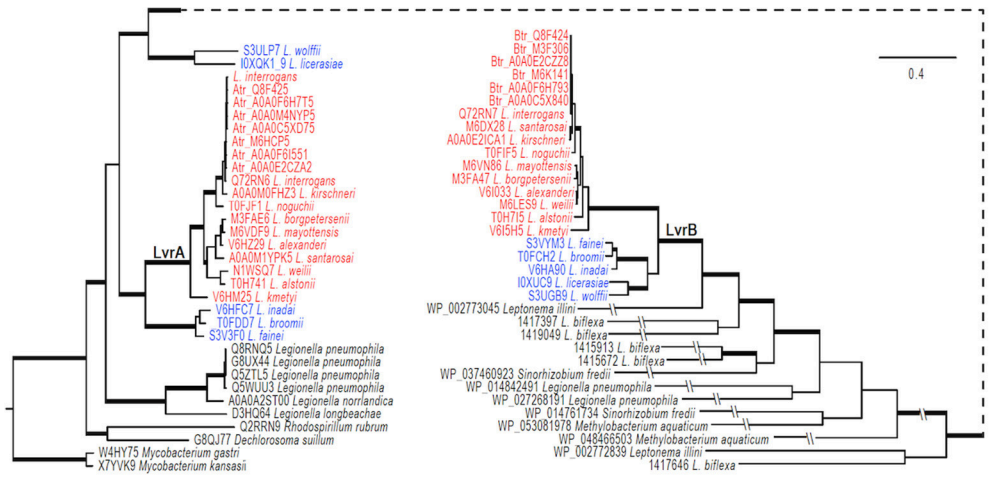


FIGURE 6 | Evolution of LvrA and LvrB. Phylogenetic relationships of *lvr* genes in pathogenic *Leptospira* (indicated in red) with intermediate *Leptospira* (indicated in blue) and related two-component systems in sampled bacteria were inferred from an amino acid alignment using Bayesian approaches with models averaged parameter sets of rate matrix. The trees were rooted with sequences from two *Mycobacterium* species. The majority-rule consensus of 8001 MCMCMC-sampled trees with averaged branch length is present, and branches with strong support (BPP > 0.98) are in boldface. Bar indicates the substitutions per amino acid site. The tree is broken at a node for a better presentation, and a dashed line is used to link the node. Exceedingly long branches are foreshortened, as indicated with the symbol -//-.

protein-protein interactions, or could be involved in binding co-factors through which sensing of oxygen, light or yet cellular redox state have been demonstrated (Henry and Crosson, 2011). Distortions in the PAS central β-sheet due to ligand binding can cause quaternary changes, which are transmitted along the HK dimer interface toward the kinase transmitter domain and eventually modulate the output HK activity (Cheung and Hendrickson, 2010). In a signal-dependent way, the PAS domain of LvrA likely modulates the autokinase activity of its first HK module, according to a scenario found in canonical PAS-containing HKs (Cheung and Hendrickson, 2010). To date, only few cytosolic (soluble) HKs have been reported including the NtrBC protein in enteric bacteria, KinA in *Bacillus subtilis*, HoxJA in *Ralstonia eutropha*, TodS in *P. putida* and ThkA in *T. maritima*. PAS domains of these HKs sense environmental stimuli were found to have direct access to the cell by diffusion or transmission (Reitzer, 1996; Lenz and Friedrich, 1998; Phillips and Strauch, 2002; Busch et al., 2007; Gao and Stock, 2009; Yamada et al., 2009). In pathogenic bacteria, the only examples include DosS and DosT in *M. tuberculosis*; each of these harbors PAS domains that respond to low O_2 (Kumar et al., 2007). Alternatively, small molecules such as acetyl phosphate might function *in vivo* as a signal under certain metabolic conditions, by donating its phosphoryl group to certain response regulators independent of HK (Wolfe, 2010). Given the cytosolic location of the Lvr system, molecules like acetyl phosphate could serve as a potential signal, directly activating LvrB protein devoid of a PAS domain but with an N-terminal REC domain (**Figure 7**).

LvrA autophosphorylates on His218, and preferentially transfers the phosphate group to its own conserved aspartate downstream (Asp524). Alternatively, LvrA can also phosphotransfer to Asp56 on LvrB as well (**Figure 7**). This activation mode of LvrB, lacking a sensor domain of its own, is comparable to *Sphingomonas melonis* PakF, which is phosphorylated by the HK KipF during stress response (Kaczmarczyk et al., 2015). Phosphotransfer to reactive aspartate residues within the HRR regions of both LvrA and LvrB, likely controls the kinase activities of their effector HK domains and in fine modulating downstream RRs. Given the global transcriptional regulation effect found in *lvr* mutant strains (**Figure 3A**), we posit that RRs with direct transcriptional regulation capacity are likely to work downstream of Lvr. This is also supported by the fact that both LvrA and LvrB lack DNA-binding effector domains within their HRR modules.

We identified eight putative RRs harboring DNA-binding effector domains in the *L. interrogans* genome (Table S8). All of these are located in the genome adjacent to an HK gene, hence likely functioning as cognate partners in TCSs. Lvr signaling system is thus expected to interact with one or more of these TCSs, giving rise to an inherently branched pathway, ultimately interfering with those TCSs that control DNA transcription via a more complex network of phosphotransfer events. The fact that LvrA and LvrB are unusually abundant proteins, compared to the typical concentration ranges found for signaling components in the cell (Malmström et al., 2009), and that they are overexpressed during infection (**Figure 4A**), is consistent with their potential ability to cross-talk. This cross-talk could be achieved by overcoming specific paired interactions among cognate HKs and RRs, ultimately subverting the activation of one or more DNA-binding RRs.

Examples of branched signaling in bacteria have been described in a number of different pathways, including some mediated by HRRs (Garzon and Parkinson, 1996; Kaczmarczyk et al., 2015). The topology of branching in the case of

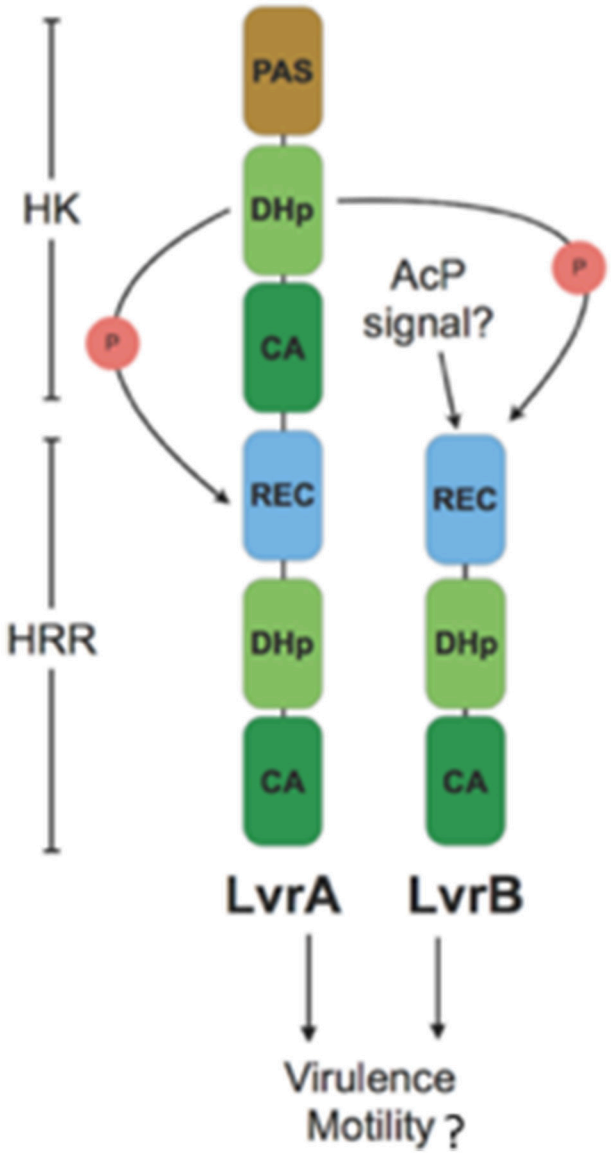


FIGURE 7 | Model for branched signaling pathway of Lvr hybrid two-component system: An unknown input signal modulates the autokinase activity of N-terminal HK module in LvrA. Upon switching to a kinase-on state, there would be phosphotransfer to its downstream HRR module, as well as to LvrB in a branched pathway. Alternatively, LvrB can be activated by a small molecule signal such as AcP (Acetyl Phosphate). After phosphotransfer events, Lvr proteins influence the expression of virulence and motility genes either by activation of putative downstream RRs or by protein-protein interactions.

Lvr is expected to correspond to a divergent cascade, with signal triggered information flow going from LvrA to LvrB to downstream effector partners or it could be directly from LvrA to other effectors. Moreover, we cannot exclude the possibility that LvrB could also be activated by alternative upstream kinases other than LvrA itself. This divergent and branched signaling flow is anticipated to allow for a particularly diverse set of adaptive responses that *Leptospira* mounts, fine-tuned to the extremely varied range of growth niches in which these spirochetes are able to live in (**Figure 7**). In our transcriptome analysis of *lvr* mutants, we observed differentially expressed genes (13%) belonging to transcriptional and signal transduction categories (Figure S2). A rich regulatory network can thus be envisaged, similar to the one controlled by the BvgA/BvgS TCS of *Bordetella pertussis*, where a multistep His-Asp-His phosphorelay occurs between different kinase domains prior to phosphorylation of the BvgA response regulator (Uhl and Miller, 1996). Therefore we cannot exclude the possibility that LvrAB directly interact with DNA-binding proteins, thus exerting the global regulatory effect (Gao et al., 2007). Further studies should be conducted to determine if the transcriptional differences observed between the WT and *lvr* mutant strains are due to interaction of LvrA/B proteins with collateral regulatory networks.

In summary, the identification of Lvr, a TCS that controls virulence and motility in pathogenic *Leptospira* unveiled the existence of a complex signaling network in this genus. To the best of our knowledge, this is the first report of a virulence-associated two-component system in this important zoonotic pathogen. The evidence of global transcriptional regulation by Lvr presented in this study allows us to speculate its role in dynamic modulation of metabolic activities and expression of virulence determinants. These new findings related to Lvr will provide us with a defined framework to identify "classical" pathogenic factors (e.g. toxins, adhesins, secretion apparatuses, etc.) under its coordinated regulation through which *Leptospira* can reprogram and adapt to the host.

AUTHOR CONTRIBUTIONS

HA, EW, AM, EG, AB, and AK: Conceived and designed the experiments; HA, EW, AM, and VB: Performed the experiments; Data Analysis and Interpretation: HA, EW, AM, SM, ZW, LS, PD, FL, JT, EG, AB, and AK; PD, GM, BA, FL, JT, MP, AB, and AK: Contributed reagents, materials, analysis tools; HA: Drafted the manuscript; EW, AM, SM, ZW, LS, PD, GM, BA, FL, JT, EG, MP, AB, and AK: Revised the paper.

FUNDING

This work was supported by grants from the National Institutes of Health (U01 AI0038752, R01 AI052473, R01 TW009504), Institut Pasteur (ACIP # A11-2010 and PTR #407), ANII Program Alianzas (ALI_1_2014_1_4982) and Program Fondo Sectorial Innovagro (FSA_1_2013_1_12557).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00045/full#supplementary-material

REFERENCES

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq–a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Asuthkar, S., Velineni, S., Stadlmann, J., Altmann, F., and Sritharan, M. (2007). Expression and characterization of an iron-regulated hemin-binding protein, HbpA, from *Leptospira interrogans* serovar Lai. *Infect. Immun.* 75, 4582–4591. doi: 10.1128/IAI.00324-07

Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., et al. (2003). Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* 3, 757–771. doi: 10.1016/S1473-3099(03)00830-2

Bourhy, P., Louvel, H., Saint Girons, I., and Picardeau, M. (2005). Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. *J. Bacteriol.* 187, 3255–3258. doi: 10.1128/JB.187.9.3255-3258.2005

Busch, A., Lacal, J., Martos, A., Ramos, J. L., and Krell, T. (2007). Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13774–13779. doi: 10.1073/pnas.0701547104

Caimano, M. J., Sivasankaran, S. K., Allard, A., Hurley, D., Hokamp, K., Grassmann, A. A., et al. (2014). A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar Copenhageni. *PLoS Pathog.* 10:e1004004. doi: 10.1371/journal.ppat.1004004

Cerveny, L., Straskova, A., Dankova, V., Hartlova, A., Ceckova, M., Staud, F., et al. (2013). Tetratricopeptide repeat motifs in the world of bacterial pathogens: role in virulence mechanisms. *Infect. Immun.* 81, 629–635. doi: 10.1128/IAI.01035-12

Cheung, J., and Hendrickson, W. A. (2010). Sensor domains of two-component regulatory systems. *Curr. Opin. Microbiol.* 13, 116–123. doi: 10.1016/j.mib.2010.01.016

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015a). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

Costa, F., Wunder, E. A. Jr., De Oliveira, D., Bisht, V., Rodrigues, G., Reis, M. G., et al. (2015b). Patterns in *Leptospira* shedding in norway rats (*Rattus norvegicus*) from brazilian slum communities at high risk of disease transmission. *PLoS Negl. Trop. Dis.* 9:e0003819. doi: 10.1371/journal.pntd.0003819

Eshghi, A., Becam, J., Lambert, A., Sismeiro, O., Dillies, M. A., Jagla, B., et al. (2014). A putative regulatory genetic locus modulates virulence in the pathogen *Leptospira interrogans*. *Infect. Immun.* 82, 2542–2552. doi: 10.1128/IAI.01803-14

Espinosa, J., Rodríguez-Mateos, F., Salinas, P., Lanza, V. F., Dixon, R., De La Cruz, F., et al. (2014). PipX, the coactivator of NtcA, is a global regulator in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2423–E2430. doi: 10.1073/pnas.1404097111

Fouts, D. E., Matthias, M. A., Adhikarla, H., Adler, B., Amorim-Santos, L., Berg, D. E., et al. (2016). What makes a bacterial species pathogenic?: comparative genomic analysis of the genus *Leptospira*. *PLoS Negl. Trop. Dis.* 10:e0004403. doi: 10.1371/journal.pntd.0004403

Gao, R., Mack, T. R., and Stock, A. M. (2007). Bacterial response regulators: versatile regulatory strategies from common domains. *Trends Biochem. Sci.* 32, 225–234. doi: 10.1016/j.tibs.2007.03.002

Gao, R., and Stock, A. M. (2009). Biological insights from structures of two-component proteins. *Annu. Rev. Microbiol.* 63, 133–154. doi: 10.1146/annurev.micro.091208.073214

Garzon, A., and Parkinson, J. S. (1996). Chemotactic signaling by the P1 phosphorylation domain liberated from the CheA histidine kinase of *Escherichia coli*. *J. Bacteriol.* 178, 6752–6758. doi: 10.1128/jb.178.23.6752-6758.1996

Goulian, M. (2010). Two-component signaling circuit structure and properties. *Curr. Opin. Microbiol.* 13, 184–189. doi: 10.1016/j.mib.2010.01.009

Graham, M. R., Smoot, L. M., Migliaccio, C. A., Virtaneva, K., Sturdevant, D. E., Porcella, S. F., et al. (2002). Virulence control in group a *Streptococcus* by a two-component gene regulatory system: global expression profiling and *in vivo* infection modeling. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13855–13860. doi: 10.1073/pnas.202353699

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Henry, J. T., and Crosson, S. (2011). Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu. Rev. Microbiol.* 65, 261–286. doi: 10.1146/annurev-micro-121809-151631

Johnson, R. C., and Harris, V. G. (1967). Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *J. Bacteriol.* 94, 27–31.

Kaczmarczyk, A., Hochstrasser, R., Vorholt, J. A., and Francez-Charlot, A. (2015). Two-tiered histidine kinase pathway involved in heat shock and salt sensing in the general stress response of *Sphingomonas melonis* Fr1. *J. Bacteriol.* 197, 1466–1477. doi: 10.1128/JB.00019-15

Kassegne, K., Hu, W., Ojcius, D. M., Sun, D., Ge, Y., Zhao, J., et al. (2014). Identification of collagenase as a critical virulence factor for invasiveness and transmission of pathogenic *Leptospira* species. *J. Infect. Dis.* 209, 1105–1115. doi: 10.1093/infdis/jit659

Ko, A. I., Galvao Reis, M., Ribeiro Dourado, C. M., Johnson, W. D. Jr., and Riley, L. W. (1999). Urban epidemic of severe leptospirosis in Brazil. *Salvador Leptospirosis* Study Group. *Lancet* 354, 820–825. doi: 10.1016/S0140-6736(99)80012-9

Ko, A. I., Goarant, C., and Picardeau, M. (2009). *Leptospira:* the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* 7, 736–747. doi: 10.1038/nrmicro2208

Kumar, A., Toledo, J. C., Patel, R. P., Lancaster, J. R. Jr., and Steyn, A. J. (2007). *Mycobacterium tuberculosis* DosS is a redox sensor and DosT is a hypoxia sensor. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11568–11573. doi: 10.1073/pnas.0705054104

Lambert, A., Wong Ng, J., and Picardeau, M. (2015). Gene inactivation of a chemotaxis operon in the pathogen *Leptospira interrogans*. *FEMS Microbiol. Lett.* 362, 1–8. doi: 10.1093/femsle/fnu054

Lenz, O., and Friedrich, B. (1998). A novel multicomponent regulatory system mediates H_2 sensing in *Alcaligenes eutrophus*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12474–12479. doi: 10.1073/pnas.95.21.12474

Liu, K., Warnow, T. J., Holder, M. T., Nelesen, S. M., Yu, J., Stamatakis, A. P., et al. (2012). SATe-II: very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. *Syst. Biol.* 61, 90–106. doi: 10.1093/sysbio/syr095

Liu, T., Ramesh, A., Ma, Z., Ward, S. K., Zhang, L., George, G. N., et al. (2007). CsoR is a novel *Mycobacterium tuberculosis* copper-sensing transcriptional regulator. *Nat. Chem. Biol.* 3, 60–68. doi: 10.1038/nchembio844

Louvel, H., Betton, J. M., and Picardeau, M. (2008). Heme rescues a two-component system *Leptospira* biflexa mutant. *BMC Microbiol.* 8:25. doi: 10.1186/1471-2180-8-25

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Malmström, J., Beck, M., Schmidt, A., Lange, V., Deutsch, E. W., and Aebersold, R. (2009). Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature* 460, 762–765. doi: 10.1038/nature08184

Mascher, T., Helmann, J. D., and Unden, G. (2006). Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol. Mol. Biol. Rev.* 70, 910–938. doi: 10.1128/MMBR.00020-06

Miras, I., Saul, F., Nowakowski, M., Weber, P., Haouz, A., Shepard, W., et al. (2015). Structural characterization of a novel subfamily of leucine-rich repeat proteins from the human pathogen *Leptospira interrogans*. *Acta Crystallogr. D Biol. Crystallogr.* 71, 1351–1359. doi: 10.1107/S13990047150 0704X

Morero, N. R., Botti, H., Nitta, K. R., Carrión, F., Obal, G., Picardeau, M., et al. (2014). HemR is an OmpR/PhoB-like response regulator from Leptospira, which simultaneously effects transcriptional activation and repression of key haem metabolism genes. *Mol. Microbiol.* 94, 340–352. doi: 10.1111/mmi.12763

Nascimento, A. L., Ko, A. I., Martins, E. A., Monteiro-Vitorello, C. B., Ho, P. L., Haake, D. A., et al. (2004). Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* 186, 2164–2172. doi: 10.1128/JB.186.7.2164-2172.2004

Noble, W. S. (2009). How does multiple testing correction work? *Nat. Biotechnol.* 27, 1135–1137. doi: 10.1038/nbt1209-1135

Phillips, Z. E., and Strauch, M. A. (2002). *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell. Mol. Life Sci.* 59, 392–402. doi: 10.1007/s00018-002-8431-9

Pinne, M., Matsunaga, J., and Haake, D. A. (2012). Leptospiral outer membrane protein microarray, a novel approach to identification of host ligand-binding proteins. *J. Bacteriol.* 194, 6074–6087. doi: 10.1128/JB.01119-12

Powell, A. J., Conant, G. C., Brown, D. E., Carbone, I., and Dean, R. A. (2008). Altered patterns of gene duplication and differential gene gain and loss in fungal pathogens. *BMC Genomics* 9:147. doi: 10.1186/1471-2164-9-147

Reis, R. B., Ribeiro, G. S., Felzemburgh, R. D., Santana, F. S., Mohr, S., Melendez, A. X., et al. (2008). Impact of environment and social gradient on *Leptospira* infection in urban slums. *PLoS Negl. Trop. Dis.* 2:e228. doi: 10.1371/journal.pntd.0000228

Reitzer, L. J. (1996). "Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine, and D-alanine," in *Escherichia coli and Salmonella: Cellular and Molecular Biology,* eds F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, M. Riley, M. Schaechter, and H. E. Umbarger (Washington, DC: ASM Press), 391–407.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542. doi: 10.1093/sysbio/sys029

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Trapnell, C., Pachter, L., and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25, 1105–1111. doi: 10.1093/bioinformatics/btp120

Uhl, M. A., and Miller, J. F. (1996). Central role of the BvgS receiver as a phosphorylated intermediate in a complex two-component phosphorelay. *J. Biol. Chem.* 271, 33176–33180. doi: 10.1074/jbc.271.52.33176

Van Den Ent, F., and Löwe, J. (2006). RF cloning: a restriction-free method for inserting target genes into plasmids. *J. Biochem. Biophys. Methods* 67, 67–74. doi: 10.1016/j.jbbm.2005.12.008

Vieira, M. L., Fernandes, L. G., Domingos, R. F., Oliveira, R., Siqueira, G. H., Souza, N. M., et al. (2014). Leptospiral extracellular matrix adhesins as mediators of pathogen-host interactions. *FEMS Microbiol. Lett.* 352, 129–139. doi: 10.1111/1574-6968.12349

Wojnowska, M., Yan, J., Sivalingam, G. N., Cryar, A., Gor, J., Thalassinos, K., et al. (2013). Autophosphorylation activity of a soluble hexameric histidine kinase correlates with the shift in protein conformational equilibrium. *Chem. Biol.* 20, 1411–1420. doi: 10.1016/j.chembiol.2013.09.008

Wolfe, A. J. (2010). Physiologically relevant small phosphodonors link metabolism to signal transduction. *Curr. Opin. Microbiol.* 13, 204–209. doi: 10.1016/j.mib.2010.01.002

Wuichet, K., Cantwell, B. J., and Zhulin, I. B. (2010). Evolution and phyletic distribution of two-component signal transduction systems. *Curr. Opin. Microbiol.* 13, 219–225. doi: 10.1016/j.mib.2009.12.011

Wunder, E. A. Jr., Figueira, C. P., Benaroudj, N., Hu, B., Tong, B. A., Trajtenberg, F., et al. (2016). A novel flagellar sheath protein, FcpA, determines filament coiling, translational motility and virulence for the *Leptospira spirochete*. *Mol. Microbiol.* 101, 457–470. doi: 10.1111/mmi.13403

Yamada, S., Sugimoto, H., Kobayashi, M., Ohno, A., Nakamura, H., and Shiro, Y. (2009). Structure of PAS-linked histidine kinase and the response regulator complex. *Structure* 17, 1333–1344. doi: 10.1016/j.str.2009.07.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 19 January 2017
doi: 10.3389/fcimb.2017.00010

Genome-Wide Transcriptional Start Site Mapping and sRNA Identification in the Pathogen *Leptospira interrogans*

Anna Zhukova[1], Luis Guilherme Fernandes[2], Perrine Hugon[2,3], Christopher J. Pappas[2,4], Odile Sismeiro[5], Jean-Yves Coppée[5], Christophe Becavin[1], Christophe Malabat[1], Azad Eshghi[2†], Jun-Jie Zhang[6], Frank X. Yang[6] and Mathieu Picardeau[2]*

[1] *Bioinformatics and Biostatistics Hub, Institut Pasteur, C3BI, Paris, France,* [2] *Biology of Spirochetes Unit, Institut Pasteur, Paris, France,* [3] *Mutualized Microbiology Platform, Institut Pasteur, Pasteur International Bioresources Network, Paris, France,* [4] *Department of Biology, Manhattanville College, Purchase, NY, USA,* [5] *CITECH, Institut Pasteur, Plate-forme Transcriptome et Epigenome, Pole Biomics – CITECH, Paris, France,* [6] *Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, USA*

Leptospira are emerging zoonotic pathogens transmitted from animals to humans typically through contaminated environmental sources of water and soil. Regulatory pathways of pathogenic *Leptospira* spp. underlying the adaptive response to different hosts and environmental conditions remains elusive. In this study, we provide the first global Transcriptional Start Site (TSS) map of a *Leptospira* species. RNA was obtained from the pathogen *Leptospira interrogans* grown at 30°C (optimal *in vitro* temperature) and 37°C (host temperature) and selectively enriched for 5′ ends of native transcripts. A total of 2865 and 2866 primary TSS (pTSS) were predicted in the genome of *L. interrogans* at 30 and 37°C, respectively. The majority of the pTSSs were located between 0 and 10 nucleotides from the translational start site, suggesting that leaderless transcripts are a common feature of the leptospiral translational landscape. Comparative differential RNA-sequencing (dRNA-seq) analysis revealed conservation of most pTSS at 30 and 37°C. Promoter prediction algorithms allow the identification of the binding sites of the alternative sigma factor sigma 54. However, other motifs were not identified indicating that *Leptospira* consensus promoter sequences are inherently different from the *Escherichia coli* model. RNA sequencing also identified 277 and 226 putative small regulatory RNAs (sRNAs) at 30 and 37°C, respectively, including eight validated sRNAs by Northern blots. These results provide the first global view of TSS and the repertoire of sRNAs in *L. interrogans.* These data will establish a foundation for future experimental work on gene regulation under various environmental conditions including those in the host.

Keywords: leptospirosis, spirochetes, promoter, transcription factors, RNA

OPEN ACCESS

Edited by:
Rey Carabeo,
Washington State University, USA

Reviewed by:
Philip E. Stewart,
Rocky Mountain Laboratories (NIAID-NIH), USA
Jarlath E. Nally,
United States Department of Agriculture, USA
Melissa Jo Caimano,
University of Connecticut Health Center, USA
James Matsunaga,
University of California, Los Angeles, USA
Haritha Adhikarla,
Yale University, USA

***Correspondence:**
Mathieu Picardeau
mpicard@pasteur.fr

†Present Address:
Azad Eshghi,
Faculty of Dentistry, University of Toronto, Toronto, Canada

Received: *14 November 2016*
Accepted: *06 January 2017*
Published: *19 January 2017*

Citation:
Zhukova A, Fernandes LG, Hugon P, Pappas CJ, Sismeiro O, Coppée J-Y, Becavin C, Malabat C, Eshghi A, Zhang J-J, Yang FX and Picardeau M (2017) Genome-Wide Transcriptional Start Site Mapping and sRNA Identification in the Pathogen Leptospira interrogans. Front. Cell. Infect. Microbiol. 7:10. doi: 10.3389/fcimb.2017.00010

INTRODUCTION

Pathogenic *Leptospira* spp. are the etiologic agents of leptospirosis, a disease manifesting as a wide range of clinical symptoms. A recent study estimates that more than one million severe cases of leptospirosis occur annually, including 60,000 deaths (Costa et al., 2015). Rats are asymptomatic reservoirs of pathogenic *Leptospira* spp. and contribute to the transmission cycle of the bacteria via bacterial shedding through the urinary tract to environmental sources. Other mammalian species, wild, and domestic, can also serve as reservoirs and present a range of mild to fatal disease manifestations. *Leptospira* are typically transmitted to humans by exposure to environmental surface water that is contaminated with the urine of infected animals. Leptospirosis has emerged as a major public health problem, especially in the developing world, due to global climate changes and urban sprawl.

Our current understanding of the virulence mechanisms and more generally the biology of pathogenic *Leptospira* remains largely unknown, partly due to the lack of efficient genetic tools and fastidious *in vitro* culturing of pathogenic *Leptospira* spp. (Ko et al., 2009). The transmission cycle of *Leptospira* exposes the bacteria to drastically different environments and *Leptospira* must be able to adapt to such disparities to retain viability. Adaptive responses of *Leptospira interrogans* have been analyzed by whole-genome microarrays to determine global changes in transcript levels of *L. interrogans* in response to interaction with phagocytic cells (Xue et al., 2010), temperature (Lo et al., 2006; Qin et al., 2006), osmolarity (Matsunaga et al., 2007), iron depletion (Lo et al., 2010), and serum exposure (Patarakul et al., 2010), which are relevant to changes that occur during infection. These transcriptome studies have shown that *Leptospira* spp. are capable of responding to a diverse array of environmental signals. However, the molecular mechanisms of bacterial adaptation and regulatory networks remain unknown.

In a recent study, high-throughput RNA sequencing of *L. interrogans* serovar Copenhageni cultivated within dialysis membrane chambers (DMCs) implanted into the peritoneal cavities of rats allowed the identification of 11 putative small non-coding RNAs (sRNAs) whose functions remain to be determined (Caimano et al., 2014). Other potential regulatory non-coding RNAs identified in *Leptospira* spp. include an RNA thermometer (Matsunaga et al., 2013) and riboswitches (Ricaldi et al., 2012; Fouts et al., 2016; Iraola et al., 2016). In addition to transcription factors, *Leptospira* species have several alternative sigma factors that are known to be important for environmental adaptation and bacterial virulence in other bacteria (Kazmierczak et al., 2005), such as σ^{54} (σ^{N}, RpoN) involved in nitrogen utilization and many cellular and environmental responses, σ^{28} (σ^{F}, FliA) involved in flagella gene expression, and several extracytoplasmic function (ECF) sigma factors σ^{24} (σ^{E}) involved in regulation of membrane and periplasmic stress.

To improve genome annotation and promote our understanding of *L. interrogans* gene structures and RNA-based regulation, we present here a transcriptional map of the *L. interrogans* genome including the characterization of primary transcription start sites (TSS), alternative TSS, operon organization, and specific DNA sequence motifs located in promoter sequences. Deep RNA sequencing also contributes to the identification of sRNAs among which some were further experimentally validated. This approach, selective for the 5′ ends of primary transcripts, has been used for transcriptome analysis, TSS determination, and regulatory RNA discovery in many other pathogenic bacteria, including *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* (Sahr et al., 2012; Wurtzel et al., 2012; Cortes et al., 2013). These results should improve our knowledge of gene regulatory circuits that control gene expression in this emerging zoonotic pathogen.

MATERIAL AND METHODS

Strains, Culture Conditions, and RNA Isolation

L. interrogans serovar Manilae strain L495 was grown aerobically at 30°C in Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (Ellinghausen and McCullough, 1965) with shaking at 100 rpm to mid log phase ($\sim 1 \times 10^8$ Leptospira/ml) then shifted to 37°C or maintained at 30°C for 18 h. Total RNA was extracted from triplicate cultures as previously described (Pappas and Picardeau, 2015). The quality of RNA was assessed using a Bioanalyzer system (Agilent). Ribosomal RNA was depleted by specific rRNA modified capture hybridization approach ("MicrobExpress" kit, AM1905, Ambion), allowing an enrichment of messenger RNA (mRNA).

Construction of CDNA Libraries for Illumina Sequencing

rRNA depleted RNA samples from triplicate exponential cultures for each of the studied temperatures (30 and 37°C) were pooled and divided into four similar fractions.

Directional cDNA libraries for whole-transcriptome sequencing were constructed by using the TruSeq Stranded RNA LT Sample Prep kit (Illumina) from enriched non-rRNAs that were fragmented by using a Fragmentation kit from Ambion, and purified on RNeasy MinElute columns (Qiagen). Fragments of cDNA of 150 bp were purified from each library and quality was confirmed on a Bioanalyzer apparatus (Agilent).

To discriminate the primary transcripts from those with processed 5′ ends for TSS mapping, the enriched non-rRNAs was (1) untreated or (2) treated with Terminator 5′ Phosphatase Dependent Exonuclease (TEX) (Epicentre), or (3) treated with TEX and then treated with tobacco acid pyrophosphatase (TAP). cDNA librairies were prepared as described for the RNA-sequencing analysis but omitting the RNA size-fractionation step. First-strand cDNA synthesis was performed by ligation with an excess of 5′ adapter (Illumina TruSeq Small RNA kit) and by reverse transcription using a random primer (RPO primer: 5′-CCTTGGCACCCGAGAATTCCANNNNNN-3′). The cDNAs were size-fractioned within the range of 120 to 250 bp on agarose gels and purified using a QIAquick Gel Extraction Kit (Qiagen). The resulting cDNAs were PCR amplified for 14 cycles using the Illumina primer RP1, and one of the indexed primers

(Illumina TruSeq Small RNA kit). The resulting PCR products were purified with Agencourt AMPure Beads XP (Beckman).

Quality of the eight cDNA libraries were confirmed on a Bioanalyser (Agilent) and each library was sequenced in single-end mode for 51 bp, using an Illumina HiSeq2500 instrument (Illumina). Reads were cleaned from adapter sequences with AlienTrimmer (Criscuolo and Brisse, 2013) (version 0.4.0) and duplicates and low quality reads using PRINSEQ (Schmieder and Edwards, 2011) (version 0.20.3). The reads were aligned to the reference genome of *L. interrogans* serovar Manilae strain L495 (total genome size of 4,614,703 bases, GC% of 34.99, number of contigs is 88, and 4261 annotated coding sequences) downloaded from MaGe platform (Vallenet et al., 2013). The alignment was performed by Rockhopper software (McClure et al., 2013), allowing 5% of read length mismatches, and using 35% of read length as minimal seed. The produced alignments were filtered to remove data with 0 scores, sorted and indexed with SAMTools (Li et al., 2009). Coverage graphs representing the numbers of mapped reads per nucleotide were generated based on the sorted reads using BEDTools (Li et al., 2009; Quinlan, 2014). On each coverage graph the upper quartile normalization (Bullard et al., 2010) was performed. To restore the original data range, each graph was then multiplied by the median of upper quartiles of all graphs corresponding to the selected temperature.

After quality trimming and duplicate removal, the TSS libraries yielded a total of 1,805,824 (out of which 1,444,131 mapped) sequence reads for the 30-TEX(−)TAP(−) library, 2,128,271 (out of which 1,689,819 mapped) sequence reads for the 30-TEX(−)TAP(+) library, 1,209,046 (out of which 986,071 mapped) sequence reads for the 30-TEX(+)TAP(+) library, 1,767,042 (out of which 1,262,780 mapped) sequence reads for the 37-TEX(−)TAP(−),1,720,339 (out of which 1,169,801 mapped) sequence reads for the 37-TEX(−)TAP(+) library, and 1,010,887 (out of which 761,737 mapped) sequence reads for the 37-TEX(+)TAP(+) library. The RNA-seq librairies yielded a total of 1,256,867 (out of which 1,150,740 mapped) and 1,495,434 (out of which 1,371,362 mapped) sequence reads at 30 and 37°C, respectively, after quality trimming and duplicates removal. The amount of reads mapping to rRNA were <1% for TSS libraries and ranged between 7 and 11% for the RNA-seq libraries.

TSS Identification and Classification

TSS were identified independently from differential RNA-sequencing (dRNA-seq) data of cultures grown at 30 and 37°C. Potential TSS were identified at the positions where all of the following conditions were met [eL(i) is the coverage at position i in the graph L]:

(i) $e_{\text{minusTEX}(-)\text{TAP}(+)}(i) \geq$ threshold (average of 3rd percentiles of normalized TEX(+)TAP(+), TEX(−)TAP(−), TEX(−)TAP(+) graphs)

(ii) Coverage change $e_{\text{TEX}(+)\text{TAP}(+)}(i) - e_{\text{TEX}(+)\text{TAP}(+)}(i-1) \geq$ threshold (same as above)

(iii) Factor of coverage change: $e_{\text{TEX}(+)\text{TAP}(+)}(i) / e_{\text{TEX}(+)\text{TAP}(+)}(i-1) \geq$ threshold (1.5)

(iv) Enrichment factor: $e_{\text{TEX}(+)\text{TAP}(+)}(i) / e_{\text{TEX}(-)\text{TAP}(-)}(i) \geq$ threshold (1.5).

TSS candidates within five nts from each other were clustered together, and in each cluster a TSS with the strongest coverage in TEX(+)TAP(+) graph was selected as the representative TSS.

Following Dugar et al. (2013), each TSS was classified as a gene TSS (gTSS), an internal TSS (iTSS), an antisense TSS (asTSS), or an orphan (oTSS) if it could not be assigned to any of the previous classes. A TSS was classified as gTSS if it was located ≤300 bp upstream of a gene. The TSS with the strongest expression values (maximum peak height) among gTSS of a gene was classified as primary (pTSS), the rest of the gTSS that were assigned to the same gene were classified as secondary TSS (sTSS). iTSS were located within an annotated gene on the sense strand and asTSS were located inside a gene or within ≤100 bp on the antisense strand. Integrative Genomics Viewer (IGV) (Robinson et al., 2011) was used to visualize the reads and location of TSS.

The clusters of orthologous groups (COG) (Tatusov, 1997) annotations of the mRNA of *L. interrogans* serovar Manilae strain L495 are available on the MaGe platform (Vallenet et al., 2013). We compared the distribution of COG classes in leaderless mRNA (whose pTSS are located between 0 and 10 nts) in comparison to genome-wide expected probabilities. To calculate the significance of leaderlessness for each COG category the Fisher exact test was used [SciPy library (Oliphant, 2007) for Python] with the following data: in the contingency table, the genes with a detected pTSS were divided into leaderless and others on the one hand, and those that belong to the selected COG category and belong to another category on the other hand. The null hypothesis was that leaderless and non-leaderless genes are equally likely to belong to the selected COG category. A $P \leq 0.05$ indicated strong evidence against the null hypothesis.

Motif Detection in Promoter Sequences

For the genes with pTSS and a 5′UTR of at least 6 nucleotides, we looked for Shine-Dalgarno (SD) sequences upstream of the start codon. Following the procedure described by Noguchi et al. (2008), we considered the nine hexamers derived from the sequence G(A/T)(A/T)AGGAGGT(G/A)ATC (complementary to a tail of 16S rRNA) as the potential SD motifs. For each gene we selected the upstream region of the start codon of up to 30 nucleotides long and looked for perfect matches or for 1-base mismatch of these nine motifs. Using the detected sequences, we constructed a position weight matrix (PWM) for each motif. Then for each gene we selected a sequence with the highest score $max_{m,j}\left[\omega_m \times \sum_{i=1}^{6} log\left(\frac{p_m(x_{i,j})}{q(x_{i,j})}\right)\right]$, where ω_m is a frequency of a motif m,$x_{i,j}$ is an i^{th} nucleotide of a hexamer j, $p_m(x_{i,j})$ is a frequency of $x_{i,j}$ at a position i of a PWM for a motif m, and $q(x_{i,j})$ is a background frequency of $x_{i,j}$ calculated from a GC content of the genome.

We extracted the 80 nucleotides upstream of the identified pTSS in *L. interrogans*, and performed motif discovery in these sequences using the MEME algorithm implemented in the MEME suite version 4.10.1 (Bailey et al., 2009). We looked for motifs of minimal length five that occur zero or one time per sequence, and are found in at leasts two sequences. We then compared motifs against the Swiss Regulon *Escherichia coli*

motifs database (Pachkov et al., 2013), and against CollecTF, a database of transcription factor binding sites (TFBS) in the Bacteria domain (Kiliç et al., 2014). The comparison was performed with Tomtom, a motif comparison tool (Gupta et al., 2007) from the MEME suite. We have also scanned for the bacterial TFBS motifs and for the *E. coli* regulatory motifs in the 80 nucleotides upstream of the identified *L. interrogans* pTSS, using FIMO tool (Gupta et al., 2007; Grant et al., 2011) from the MEME suite.

For the prediction of promoter sequences for the housekeeping sigma factors sigma70, and alternative sigma factors sigma28 and sigma24, the following matrix and spacer between −35 and −10 (in parenthesis) were used: Matrix.18_15_13_2_1.5 (13–19) for sigma70, Matrix.15.13.11.8.5.d.NC (13–15) for sigma28, and Matrix.15.13.15.5.8.d.NC (15–20) for sigma 24 (matrix resource: http://www.ccg.unam.mx/Computational_Genomics/PromoterTools/). The 80 nucleotide sequences upstream TSSs were then subjected to prediction of the presence of each sigma factor-type promoter sequence using PromoterHunter software (http://www.phisite.org/main/index.php?nav=tools&nav_sel=hunter). For the prediction of sigma54 promoter sequences, the PATSER program (Hertz and Stormo, 1999) was used to search against 80 nucleotide sequences upstream of the identified pTSS. The weight matrix of the −24/−12 sigma54-type promoter consensus sequence used in this study was based on a set of 186 RpoN-dependent promoters from different bacterial species (Barrios et al., 1999). The actual scores for the sequences were determined from the weight matrix. The higher the score, the higher the specificity. A cutoff of score >4.0 was chosen as a potential sigma54 promoter.

Operon Prediction

Operon detection was performed using software Rockhopper (McClure et al., 2013) on the total RNAseq data at 30 and 37°C. Rockhopper detects operons using a naive Bayes classifier based on prior operon probabilities, intergenic distance, and correlation of gene expression across RNA-seq experiments. Potential pTSS was identified for each operon as the pTSS detected on dRNA-seq data (see above) for the first gene of the operon. For operons with no pTSS detected on dRNA-seq data, the value identified by Rockhopper on the total RNAseq data (in the majority of cases equal to the start of the first operon gene) was used.

Putative sRNA Prediction

Putative sRNA detection was performed using software Rockhopper (McClure et al., 2013) on the total RNAseq data at 30 and 37°C. Among the transcripts identified by Rockhopper as predicted RNA, those of the length ≥50 nucleotides were kept. For each sRNA, potential pTSS were identified following the procedure described above, and potential small coding sequences were detected using any of the start codons ATG, TTG, GTG, and the stop codons TAA, TAG, TGA. For each putative sRNA, a search for matching families in Rfam database (Nawrocki et al., 2015) was performed via RESTful interface using urllib2 library for Python.

The secondary structure was predicted for each putative sRNA sequence using UNAFold (Markham and Zuker, 2008; version 3.8). Rho-independent terminator (RIT) sites were detected at positions −25 to 200 nucleotides of stop codon of each putative sRNA using Arnold software (Naville et al., 2011). We filtered out the RIT sites with values of Gibbs free energy of more than −4 kcal/mol. Putative sRNA were classified into the following categories: antisense CDS (sRNA located on an opposite strand to a coding sequence), antisense 5′UTR (sRNA located on an opposite strand to the 5′UTR of a coding sequence), antisense 3′UTR (sRNA located on an opposite strand to the 3′UTR of a coding sequence), and IGR (sRNA located in an intergenic region). Manual inspection and curation of sRNA was performed with IGV.

5′-RACE

L. interrogans total RNA was prepared from cultures grown in EMJH at 30°C at exponential growth as previously described (Pappas and Picardeau, 2015) and subjected to 5′ rapid amplification of cDNA ends (RACE) with the 5′ RACE system from Invitrogen, according to the manufacturer's instructions. The gene-specific primers for reverse transcription reactions and generation of 5′ RACE amplicons are listed in **Supplementary Table 1**. PCR products were then cloned in pCR2.1-TOPO (In vitrogen) and plasmid DNA was isolated from 5 ml of overnight culture of *E. coli* using Qiagen miniprep kit (Qiagen). Plasmids were then sequenced by Eurofins.

Northern Blot

To confirm the expression and size of putative sRNA, 2 μg of total RNA extracted from *L. interrogans* serovar Manilae were mixed together with one volume of denaturing loading buffer containing 95% formamide (Thermo Fisher), incubated at 95°C for 5 min and then placed on ice. Samples were separated by 8 M urea polyacrylamide gel (concentration ranging from 5 to 10%) in TBE buffer, along with an RNA ladder (Euromedex), for 1 h at 25 mA. The RNA integrity of samples following migration was evaluated by ethidium bromide staining (0.5 μg/mL). Gels were then transferred onto Hybond N+ membranes (Amersham) using a Criterion Blotter in TBE buffer for 1 h at 50 V. RNA molecules were crosslinked to the membranes by UV irradiation (0.51 J/cm^2) and pre-hybridized with 10 mL of ULTRAhyb hybridization buffer (Thermo Fisher) for 1 h at 42°C in a rotating chamber; then, 2 μL of 10 μM 5′biotinylated oligo DNA probe (**Supplementary Table 2**) were added and hybridization proceeded for 14 h. Membranes were washed twice in 2X SSC and 0.1% SDS and then twice in 0.1X SSC and 0.1% SDS. Hybridized probes were visualized by incubation with horseradish peroxidase-conjugated streptavidin and chemiluminescent substrate (Thermo Fisher), followed by film exposure.

Availability of Supporting Data

The raw data files for the RNA-seq experiment are deposited in the Gene Expression Omnibus (GEO) database from NCBI (Edgar et al., 2002), Gene accession GSE92976. Additionally, the genome files of *L. interrogans* serovar Manilae strain L495 used

for analysis of RNA-seq data are available in MicroScope (http://www.genoscope.cns.fr/agc/microscope/home/index.php).

RESULTS

To obtain an overview of the *L. interrogans* transcriptome, the pathogen was grown at 30°C for optimal *in vitro* growth and at 37°C to mimic the host environment and to promote the expression of genes important during the infection.

RNA-seq data of the most abundant transcripts showed that lipoproteins-encoding genes *lipL32*, *lipL21*, *lipL41*, *loa22*, and *lipL36*, 30S and 50S ribosomal subunit proteins-encoding genes, and flagellin-encoding genes were the most highly expressed genes in *L. interrogans*, which concurs with previous transcriptional and translational analyses (Lo et al., 2006; Malmström et al., 2009). Additionally, heat shock protein-encoding genes *groS* (LMANv2_150128), *groEL* (LMANv2_150129), *hsp15* (LMANv2_380017), and *hsp15*-like (LMANv2_380018) were up-regulated (two- to three-fold increase in transcript levels) by temperature upshift (**Supplementary Table 3**). Together, these results indicate that RNA preparations and temperature shift experiments were performed in a manner acceptable for subsequent transcriptome analysis. Interestingly, a 92-nucleotide gene (LMANv2_330026) was the second most highly expressed gene after *lipL32* at both 30 and 37°C. The conservation of this small gene in all leptospiral species suggests that it may play an important role in leptospiral physiology.

TSS Mapping

The vast majority of mRNAs are synthesized with a 5′-triphosphate group (5′ PPP), while the 5′ ends of transcripts generated through RNA processing and degradation, have a monophosphate group (5′ P) (Wurtzel et al., 2010). For TSS mapping, three libraries were carried out for each biological sample: one library was generated from RNA treated with terminator 5′ phosphate dependent exonuclease (TEX), which specifically degrades RNA species that carry a 5′ P, then enriching for transcripts that carry a 5′-PPP. A second library was generated from untreated total RNA. In the third library, the exonuclease-resistant RNA (primary transcripts with 5′PPP) was treated with TAP, which degrades 5′ PPP to 5′ P, making them accessible for 5′ end linker ligation. Comparing these libraries enables determination of putative TSSs (see Material and Methods). An increased number of sequencing reads from a 5′ end following TAP treatment is an identifier of a TSS.

Our comparative approach enabled the annotation of a total of 25,397 and 30,739 TSS at 30 and 37°C, respectively. TSSs were classified into different categories: gene TSS (gTSS), including primary TSS (pTSS) and secondary TSS (sTSS), internal TSSs (iTSS), including antisense TSSs (asTSS), and orphan TSSs that do not belong to the other categories (**Figure 1**). The genome position of all TSSs detected at 30 and 37°C is listed along with their categorization as primary, secondary, antisense, internal, or orphan TSS (**Supplementary Table 4**). Notably, one TSS can independently be assigned to more than one category. For example, within operon-like structures the pTSS of the downstream gene can also be internal to the upstream gene. In total, 2865 and 2866 pTSS of annotated genes or operons were identified in the genome of *L. interrogans* at 30 and 37°C, respectively. A total of 2437 and 3214 sTSS, defined as a TSS being located in close proximity of a pTSS but having fewer reads, were also detected at 30 and 37°C, respectively (**Supplementary Table 4**). Genes that were not assigned a TSS may be organized into operons (see below) or were not expressed at detectable levels. Thus, 72 and 87% of genes detected by RNA-seq at 30 and 37°C possess a pTSS, respecitvely, while only 17 and 43% of non-expressed genes at 30 and 37°Cwere assigned a pTSS, respectively. Approximately 22.6% of the pTSS identified are conserved at 30 and 37°C. In contrast, only 5.5% of the sTSS are conserved. When grouping together pTSS with a position within a distance of five nucleotides (±5 nt), 1360 pTSS are conserved at 30 and 37°C, thus 47.22% of the pTSS at 30°C are also found as pTSS at 37°C (**Figure 2A**).

Sequence analysis of the nucleotide composition of pTSS revealed a strong selection of the purines A (45–50%) and, to a lower extent, G (20–23%) at the +1 site (**Figures 2B,C**), which is usually required for efficient transcription initiation by RNA polymerase.

We analyzed the length distribution of the 5'UTR of the genes for which the pTSS were detected (**Figure 3**). We found a median 5′ UTR length of 91–97 nucleotides at 30 and 37°C, respectively. The majority of *L. interrogans* genes (430–450 genes) had a pTSS located within 10 bp of the translational start codon (**Figure 3**). Among those are 184 and 170 genes where the pTSS is identical to the translational start at 30 and 37°C, respectively (244 and 231 genes at 30 and 37°C, respectively, if we include pTSS at the −1 position). Considering these genes as leaderless, we analyzed the dependency between leaderlessness and COG. At both 30 and 37°C leaderless genes were underrepresented in categories C (energy production and conversion) and V (defense mechanisms), and overrepresented in category R (general function prediction only). At 30°C they were also overrepresented in H (coenzyme transport and metabolism). At 37°C leaderless genes were additionally underrepresented in N (cell motility) and overrepresented in E (amino acid transport and metabolism), F (nucleotide transport and metabolism), and G (carbohydrate transport and metabolism). In the other categories differences between representation of leaderless and leadered genes was not significant. Temperature shift did not result in any significant difference, as determined by Student's *t*-test, in the relative expression of leaderless mRNAs for specific COGs.

We selected 10 genes of known function with mapped pTSSs to verify the reliability of TSS designation by 5′ RACE experiments. There was good agreement between RACE determined and predicted TSS positions, with a maximum divergence of three nucleotides, except for one gene, *ahpC*, for which the TSS determined by RACE is located 17 nucleotides downstream from the predicted TSS (**Table 1**). We also compared our data with TSSs experimentally mapped in previous studies. The TSSs identified in *ligA* (Matsunaga et al., 2013), *groS*, and *groEL* (Ballard et al., 1993) were re-confirmed in this study, providing further validation of our TSS mapping (**Table 1**).

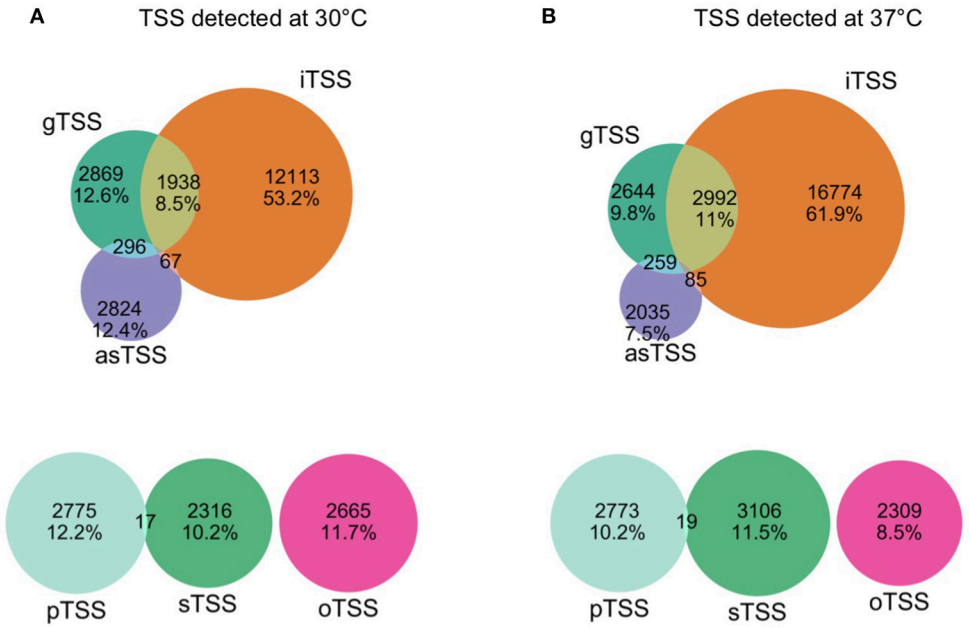


FIGURE 1 | Venn Diagram of TSS detected at 30°C (A) and 37°C **(B).** TSS were classified as gene TSS (gTSS), internal TSS (iTSS), antisense TSS (asTSS), or orphan (oTSS) (see Material and Methods). The TSS with the strongest expression values (maximum peak height) among gTSS of a gene was classified as primary (pTSS), the rest of the gTSS that were assigned to the same gene were classified as secondary (sTSS). TSS can be affiliated to multiple categories.

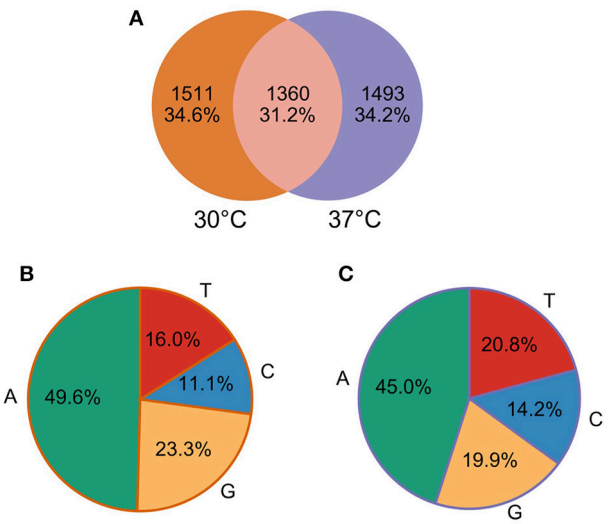


FIGURE 2 | Primary TSS (pTSS) detected at 30 and 37°C. (A) Venn Diagram of pTSS at 30 and 37°C. **(B)** Nucleotide preference at the predicted pTSS at 30°C. **(C)** Nucleotide preference at the predicted pTSS at 37°C.

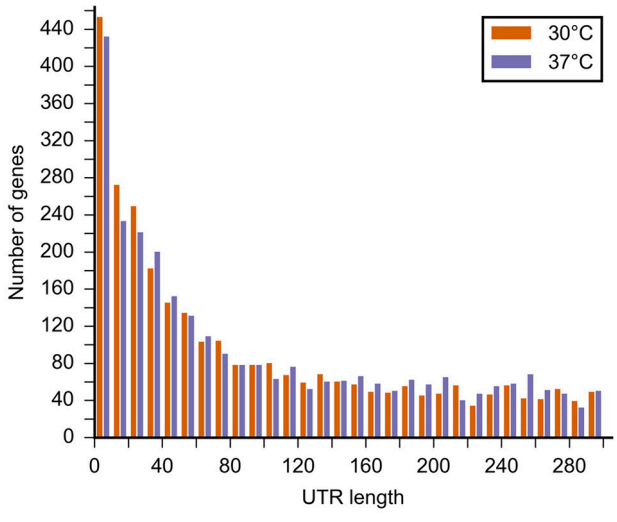


FIGURE 3 | Length distribution of the 5′UTR of the mapped pTSS at 30 and 37°C in the *L. interrogans* genome. The graph shows the length of the 5′UTR (distance from the predicted translational start to the TSS).

Operons

We defined operons in the *L. interrogans* genome as regions with continuous coverage of whole transcript reads by RNA-seq and the presence of a pTSS in the upstream sequence of coding sequences. Using these criteria 750 operons of 2–19 genes (for a total of 2181 genes) were defined at both 30 and 37°C (**Supplementary Table 4**). The average operon size of *L. interrogans* was 2.9 genes. The largest operon was 17 kb long and codes for enzymes of amino acid and cell biosynthetic pathways (*dapA-dapB-rpsB-trpA-trpB-pyrH-uppS-proS*). The second largest operon contained 16 genes (*cbiX-cbiD-cbiC-cbiT-cobI-cobJ-cobM-cobB-cobU-cobDQ-cobD*) which are involved in

TABLE 1 | Comparison of *L. interrogans* TSS identified by RNA-seq with TSS identified by 5′RACE.

Gene			Distance of TSS from CDS[a]	
			RNAseq	5′ RACE
LMANv2_60079	*flgB*	Flagellar basal body rod protein FlgB	13	12
LMANv2_110011	*dapA*	4-Hydroxy-tetrahydrodipicolinate synthase	0	0
LMANv2_150128	*groES*	Chaperone Hsp10	56	58[b]
LMANv2_370081	*fumC*	Fumarate hydratase	40	40
LMANv2_580002	*ahpC*	Peroxiredoxin	40	23
LMANv2_280031	*perR*	Ferric uptake regulator-like	1	0
LMANv2_680004	*hemO*	Heme oxygenase	22	21
LMANv2_160018	*mreB*	Actin-like component MreB	86	84
LMANv2_150111	*lipL32*	Lipoprotein LipL32	17	18
LMANv2_150129	*groEL*	Chaperone Hsp60	170	167[c]
LMANv2_630002	*ligA*	Immunoglobin-like repeats LigA	176	175[d]
LMANv2_460028	*hfq*	RNA-binding protein Hfq	146	146

[a] *Position 0 corresponds to the first nucleotide of the start codon.*
[b] *Previously identified in L. interrogans serovar Copenhageni by primer extension, see Ballard et al. (1993).*
[c] *TSS previously identified at position 61 in L. interrogans serovar Copenhageni by primer extension, see Ballard et al. (1993).*
[d] *Previously identified in L. interrogans serovar Copenhageni by 5′-RACE (Matsunaga et al., 2013).*

vitamin B12 biosynthesis. Other large operons include phage-related genes (13 genes, including genes encoding base-plate J-like and tail fiber domain proteins), and genes coding for a type II secretion system (13 genes including *gspC-gspD-gspE-gspF-gspG-gspH-gspJ-gspK-ftsA*), sialic acid biosynthesis (12 genes including *neuA1-rfb3-neuB-neuC-neuD-neuB2-neuA2*), and NADH dehydrogenase complex 1 biosynthesis (12 genes *nuoA-nuoB-nuoC-nuoD-nuoE-nuoF-nuoH-nuoK-nuoN*). The *L. interrogans* genome contains about 50 genes involved in the synthesis of the endoflagellum. Most of these genes (71%) are organized in 8 operons (from 2 to 7 genes).

For most of the downstream genes within operons, a pTSS can also be internal to the upstream genes, suggesting that the operon's genes can be transcribed through alternate promoters.

Motifs in Promoter Regions

Shine-Dalgarno sequences are defined as purine-rich hexamers complementary to the 3′-end of the 16S rRNA between 1 and 40 bp upstream of an annotated start codon. Approximately 70% of the genes with a pTSS had a predicted Shine-Dalgarno motif (**Supplementary Table 5**).

We aligned the upstream sequences of all identified pTSSs (−80 to +1) by MEME to identify potential sequence motifs in promoter regions. This resulted in the detection of two distinct sequence motifs with *P*-values below e-10 at both 30 and 37°C. These two conserved motifs, [TA]A[TA]TAGA[AG]TTGT TGAAAAATTAATTCTCCAT[CT][TG][GA]TTTC[TC]ATTT [TC]A and TGT[AG]G[GT]A[AG][TC]T[CA]C[CT]ACA[AT] [AT][TA][TAC], (i) do not have a specific nucleotide position relative to the TSS, (ii) do not resemble motifs and TFBS from the *E. coli* database, (iii) are part, at least most of them, of an intergenic repeated element, and (iv) are not found in the promoter region of the expressed gene as identified by RNA-seq (our study) and by mass spectrometry (Malmström et al., 2009). Taken together, these results suggest that these motifs may not represent DNA-binding sites (**Supplementary Table 6**).

Sigma Factors

The *L. interrogans* genome is predicted to contain 4 sigma factors: the housekeeping sigma factor σ70 (RpoD) and the alternative sigma factors σ28 (RpoF), σ54 (RpoN), and σ24 (RpoE) which provide promoter recognition specificity for the polymerase and contribute to environmental adaptation of the bacterium. We performed an *in silico* genome-wide search for putative σ70, σ28, and σ24-type promoters. The matrices used were derived from different *E. coli* promoter sequences. Given that *L. interrogans* has an AT-*rich* genome, we selected stringent criteria (see Material and Methods). We performed an *in silico* genome-wide search for putative σ70 and σ54-binding sites. A σ70-like promoter sequence (TTGACA<16–18 bp>TATAAT in *E. coli*) is found in more than 1000 *L. interrogans* genes at both 30 and 37°C (**Supplementary Table 7**). However, our analyses may fail to accurately predict this promoter sequence in the AT-*rich L. interrogans* genome and most of the identified promoter sequences most likely do not operate as σ70-binding sites. The σ54 recognizes a unique −24/−12 promoter sequence (CTGGNA<6 bp>TTGCA in *E. coli)* and is activated by enhancer-binding protein (EBP). *L. interrogans* contains two EBPs, EBP-A and EBP-B. Each EBP-σ54 pairs may respond to different signals to activate distinct transcripts of genes. A typical σ54-binding site was identified in the promoter regions of three genes encoding for putative lipoproteins (LMANv2_200027/LIC12503 and LMANv2_290065/LIC11935) and the ammonium transporter AmtB (LMANv2_310003/LIC10441) at both 30 and 37°C (**Supplementary Table 8**). Our previous EMSA results show that both recombinant σ54 and EbpA proteins are able to bind a 50-bp oligonucleotide encoding the predicted −24/−12 promoter regions of these three genes, indicating that the σ54-binding motif of *L. interrogans,* [TA][TG[CG][TAC]A<6 bp>T[GT][GC]CA, closely resembles the *E. coli* motif (Hu et al., 2017). The alternative sigma factor σ28 (sigma F) is known to regulate flagellar genes in most bacteria and predicted σ28-binding sites at position −35 and −10 from the TSS in *L. interrogans* promoter sequences comprise at least four genes coding for components of the endoflagellum (LMANv2_260046/FlaA1, LMANv2_290016/FlaB1, LMANv2_590023/FlaB4) and the flagellin-specific chaperone FliS (LMANv2_10030). Previous works have shown that σ24 (*rpoN*) is necessary for resistance to heat shock and other environmental stresses in bacteria. 469 putative σ24 binding sites are detected in the promoter regions of *L. interrogans* at both 30 and 37°C (**Supplementary Table 8**).

However, σ24 promoter sequences have a −35 region less well-conserved in phylogenetically distant bacteria, hence making prediction of binding sites in *L. interrogans* challenging.

Identification of Small Non Coding RNA (sRNA)

sRNAs are usually defined by their position in the genome relative to their target genes, with *cis*-encoded sRNAs located antisense to their target and *trans*-encoded sRNAs in intergenic regions of the genome away from their target. After manual curation, a total of 277 (pTSS annotated for 176) and 226 (pTSS annotated for 137) sRNAs were found in *L. interrogans* at 30 and 37°C, respectively; including 137 sRNAs that are conserved at both temperatures (**Figure 4A**). The predicted sRNAs displayed an average size of 101 and 98 nt at 30 and 37°C, respectively (**Supplementary Table 9**). The majority of predicted sRNAs, 168 and 147 at 30 and 37°C, respectively, were found to be located in the intergenic regions of the *L. interrogans* genome. We also identified a total of 98 and 75 antisense RNA (asRNA) candidates, at 30 and 37°C, respectively, which are located antisense inside coding regions. In addition, 29 and 19 asRNA candidates at 30 and 37°C, respectively, that are opposite to a 5′UTR or 3′UTR were detected (**Supplementary Table 9**). asRNAs overlap either with the 5′ end (14–17%), the 3′ end (9–11%), or the central region (72–77%) of the gene found on the opposite strand. The vast majority (>60%) of asRNAs overlap with genes coding hypothetical proteins; other targeted genes with a putative known function include the genes encoding lipoproteins LipL32 and LipL21 (**Figure 4B**), a TonB dependant receptor, a permease, and an anti-anti sigma factor (**Supplementary Table 9**).

Compared to the sRNA sequences in the Rfam database, few *L. interrogans* sRNAs displayed homology with well characterized sRNAs in other bacteria. Among those are a cobalamine riboswitch, tRNAs, tmRNA, also known as SsrA, RNase P RNA, and 5S rRNA. This lack of orthologs suggests these sRNAs to be novel with completely unknown function. RIT sequences were also searched at the 3′ end of the sRNAs, and 16 of the sRNAs contained typical RIT sequences, including seven that are conserved at both 30 and 37°C, indicating that the vast majority of sRNAs did not contain typical RIT (**Supplementary Table 9**). We scanned the sRNAs for the presence of small open reading frames. A total of 40 and 22 putative ORFs were identified at 30 and 37°C, ranging in size from 28 to 78 codons (**Supplementary Table 9**). The putative gene products were then examined for the presence of conserved protein domains using Blast and InterProScan. None of the deduced proteins, however, contained a known protein domain, suggesting that they may not correspond to coding regions. Secondary structures of all sRNAs were determined by minimum free energy folding and RNA shape analysis which achieved high shape probabilities in most cases (**Supplementary Table 10**).

To independently confirm the presence and size of sRNAs identified by transcriptome sequencing, Northern blotting was performed on 13 abundant sRNAs and putative sRNAs of *lipL21* and *lipL32* (**Supplementary Table 2**, **Figures 4A,B**, **5**). This analysis was carried out on cells grown to exponential phase at 30°C. Use of a non-radioactive labeling method confirmed the presence of eight of the sRNAs (**Figure 5**). For four of those, the size estimated from the transcriptome was within the size estimated from Northern blotting. In other cases, the detected transcript exceeded the size predicted by the RNA-seq data. The discrepancy in lengths may be explained by *in silico* prediction criteria. While most sRNAs displayed single and specific bands, some sRNAs exhibited additional bands which could be due to RNA processing or alternative transcription initiation (**Figure 5**).

DISCUSSION

In 2003, *L. interrogans* serovar Lai was the first *Leptospira* genome to be sequenced (Ren et al., 2003). Today, the genome sequences of hundreds of *Leptospira* strains have been determined, including representations of each of the 20 *Leptospira* species (Fouts et al., 2016). However, the difficulty of generating mutants in pathogenic strains limited the ability to analyse the wealth of information contained in these genomes and the molecular basis of leptospiral pathogenesis remains poorly understood. In this study, a combination of TSS mapping with total RNA-seq has generated a comprehensive overview of the transcriptional landscape of the pathogen *L. interrogans*.

Promoter regions are poorly characterized in *Leptospira* spp. To date, few experimentally proven TFBS have been described (Cuñé et al., 2005; Morero et al., 2014; Hu et al., 2017) in the literature and promoter prediction algorithms and *E. coli* consensus sequences of DNA motifs are not applicable to the *Leptospira* genome. Here, we annotated 2865 and 2866 pTSSs in *L. interrogans* at 30 and 37°C, respectively. Our 5′RACE results showed that our RNA-seq analysis accurately captured the TSS, confirming the accuracy of our TSS mapping. In *L. interrogans*, the majority of 5′-UTRs appear to be <80 bp, which is common for bacteria. We identified an unexpectedly high number of leaderless mRNAs, including a significant fraction of learderless mRNAs encoding products with unknown function (COG R).

We identified ∼440 leaderless mRNAs having a UTR length of <10 nucleotides, among those half of them have a UTR length of <2 nucleotides. Studies in most bacteria have typically reported only a few leaderless mRNAs as, for example, 12 leaderless genes in *L. pneumophila* (Sahr et al., 2012), 20 in *Helicobacter pylori* (Bischler et al., 2015), 23 in *Salmonella typhimurium* (Kröger et al., 2012), 30–41 in *Prochlorococcus* spp. (Voigt et al., 2014), and 57 in *Bacillus amyloliquefaciens* (Liao et al., 2015). However, an abundance of leaderless transcripts have recently been identified in the genomes of *Deinococus deserti* (1174 leaderless mRNAs) (de Groot et al., 2014), and *M. tuberculosis* (505 leaderless mRNAs) (Cortes et al., 2013). Translation of leaderless transcripts may influence translation efficiency in certain conditions and/or the half-life of trancripts (Cortes et al., 2013).

We extracted the sequences upstream of the identified TSSs and analyzed them for common motifs. This approach identified highly conserved RpoN (σ54) promoter elements.

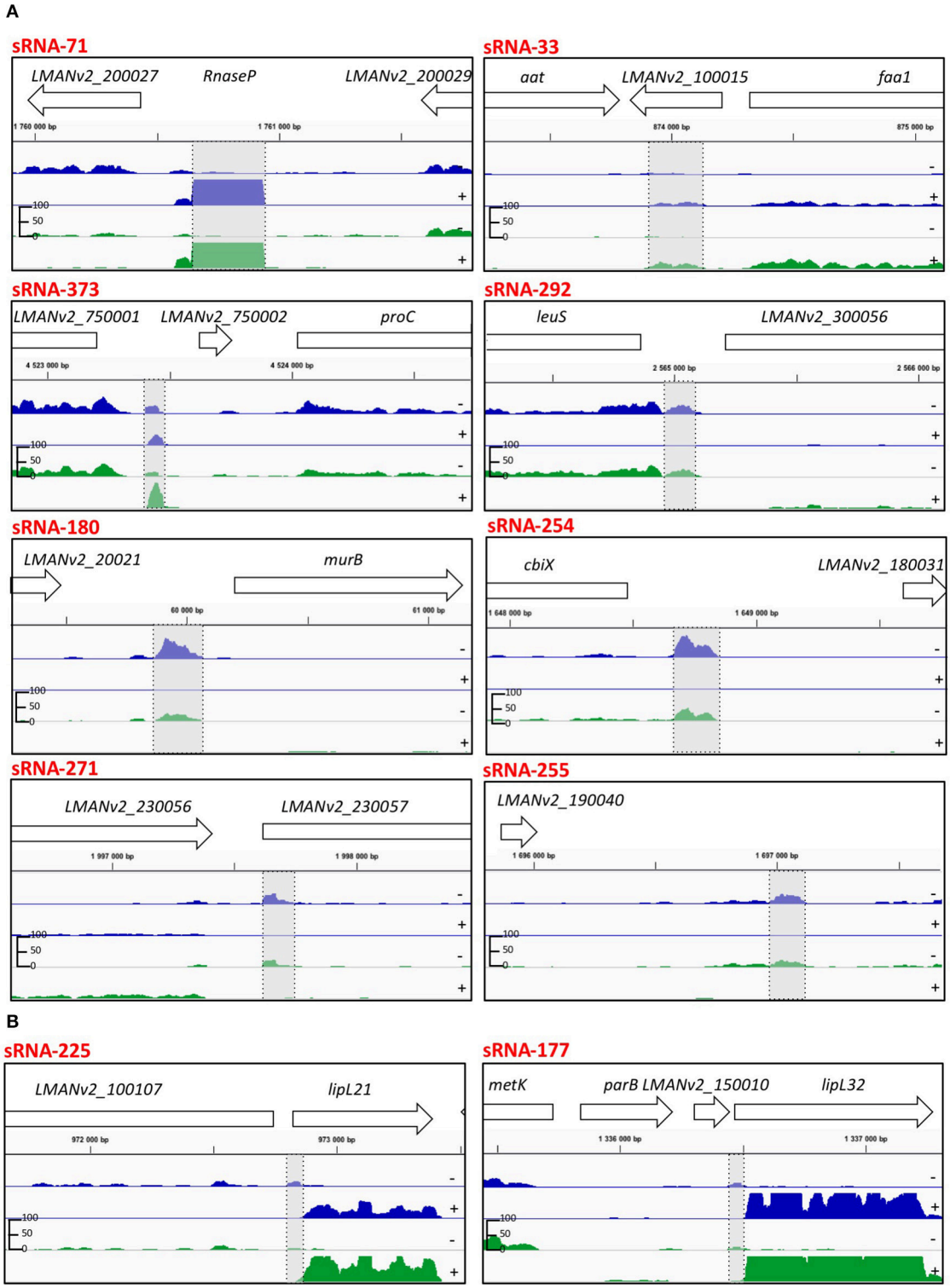


FIGURE 4 | IGB viewer of representative predicted sRNAs in the *L. interrogans* genome. (A) Candidate sRNAs that were validated by Northern blot (**Figure 5**). **(B)** Candidate sRNAs for *lipL21* and *lipL32*. Visualization of normalized mapped reads for minus (–) and plus (+) strand. Blue and green reads indicate mapped reads at 30°C and 37°C, respectively. The vertical "read count" scale is 0–100. Genomic locations and CDS are also indicated. Highlighted in gray is the predicted sRNA.

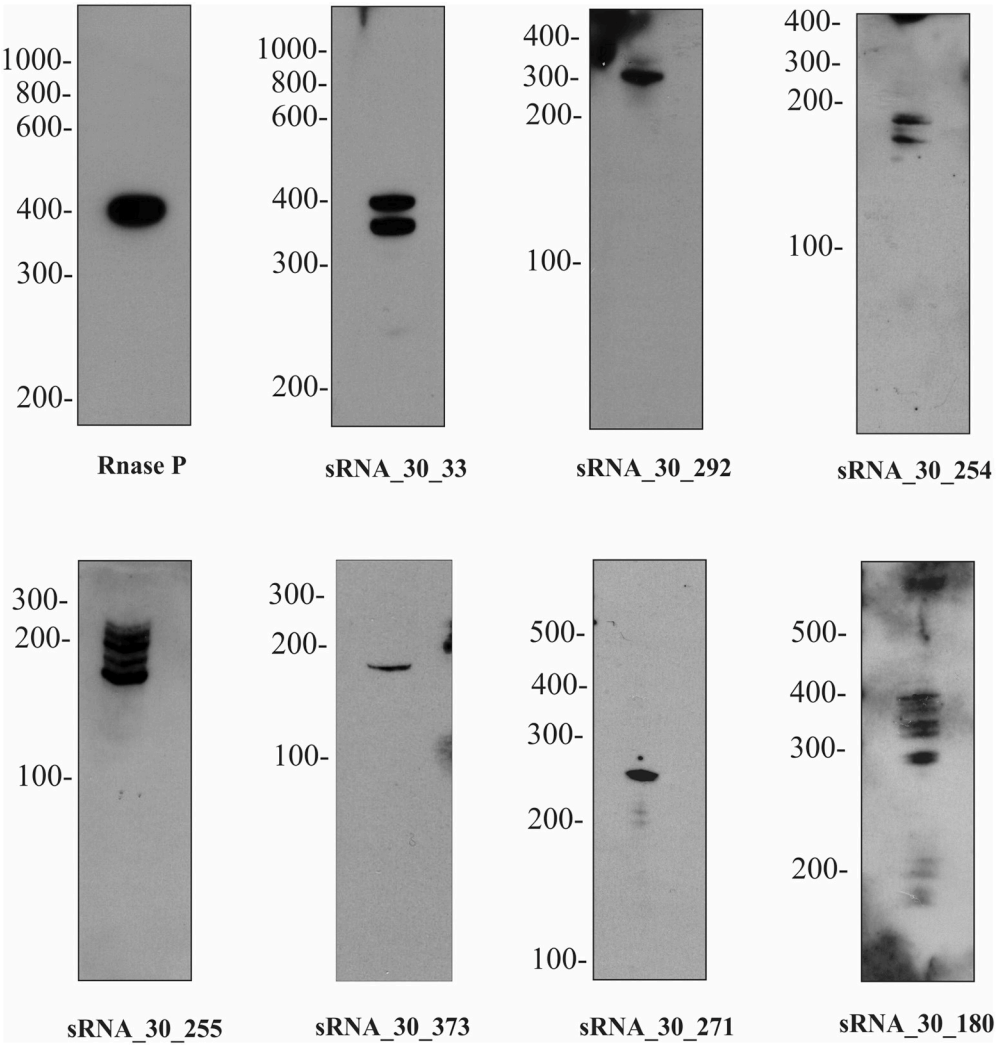


FIGURE 5 | Expression of selected sRNAs in *L. interrogans*. A subset of sRNAs identified by RNA-seq were validated by Northern blot. One representative lane was displayed for each sRNA identified. The names of sRNAs and the molecular marker were indicated on the top and on the left, respectively. For more details of each sRNA see **Figure 4A**.

However, other motifs were not identified in the promoter regions, emphasizing the relative lack of highly represented promoter motifs for *L. interrogans* transcripts. This may be due to (i) the low G+C content (35%) of the *L. interrogans* genome, (ii) the variability of the distances from the position relative to the downstream pTSS (−24/−12 or −35/−10 promoter sequences) making the identification of consensus sequences difficult, (iii) the multitude of different promoter sequences that are recognized by a variety of sigma factors and other transcriptional regulators, (iv) the inaccuracy of TSS mapping, or (v) the difficulty of predicting the *L. interrogans* promoter motifs based on *E. coli* consensus sequences. These results have significant implications for understanding the structure of promoters in *L. interrogans*. However, experimental identification of regulatory regions is necessary to improve the prediction of possible binding sites as well as to identify sequence properties that distinguish between active and weak/inactive promoters.

The genetic manipulation of pathogenic leptospires remains challenging due to its poor transformation efficiency and/or its inefficient homologous recombination machinery (Picardeau, 2015). We recently described a new strategy for creating targeted gene knockdowns in both saprophytic and pathogenic *Leptospira* spp. using TALE (Transcription Activator-Like Effector) system (Pappas and Picardeau, 2015). Since the role of a TALE protein is to repress transcription by binding directly to DNA within the promoter region of a gene (which in turn inhibits promoter recognition by RNA polymerase or by abrogating transcription initiation), identification of TSS from this study will prove helpful for designing TALEs for targeted genes in the future.

iTSSs are the most abundant category of TSSs identified by this study. The majority of operons in *L. interrogans* are complex with

internal promoters overlapping other genes that may generate multiple transcription units. Presence of iTSSs may also be due to incorrect start codon annotation or may be the result of processed derivatives of longer mRNAs (Schlüter et al., 2013). Even though a direct comparison with other TSS identification studies is not possible, it is worth mentioning that similar to *L. interrogans,* a high percentage of internal TSSs were also observed in *Borrelia burgdorferi* (Adams et al., 2016). Further experimental analysis is therefore required to validate the presence of these iTSSs.

L. interrogans has also a number of oTSSs (2636 and 2278 at 30 and 37°C, respectively, not associated with any CDS) similar to the number of pTSS. These oTSS may correspond to putative novel CDSs or sRNAs. oTSSs may also originate from missing gene information in the contig boundaries of the draft genome (see below).

We present here the first operon map of an *L. interrogans* genome paving the way to a full understanding of the complex transcriptional regulations governing the life cycle of this pathogen. A total of 750 multi-gene operons were predicted in *L. interrogans* that were mostly composed of two (57%) or three (23%) genes, as well as 10 operons that included more than ten genes (1%). These co-regulated and co-transcribed genes may allow a rapid adaptation to environmental changes, and warrant further study. *L. interrogans* contains, for example, eight operons coding for the biosynthesis of the endoflagellum. The control of expression of these genes has not been investigated in *Leptospira* spp., but in other bacteria, the genetic organization into large complex units enables a tight regulation of gene expression in a cascade that closely parallels the assembly hierarchy of the flagellar structure.

Transcriptome analysis of *L. interrogans* maintained at 30°C compared to those shifted to 37°C had a relatively minor effect on TSS and sRNA mapping. Although an increase in temperature appears to be an important signal for changes in *Leptospira* gene expression, previous studies showed that other factors such as osmolarity, iron levels, and serum exposure are also important environmental signals (Adler et al., 2011). The temperature upshift from 30 to 37°C may therefore partially mimic transfer to a mammalian species.

The use of a reference draft genome with 88 contigs can lead to mapping artifacts or missing information. Gaps represent missing genomic information and, in many cases, these gaps can coincide with genes or operons that are then disregarded in genome mapping. Use of a complete reference genome will allow a more detailed analyses of our data. Our results will also allow re-annotation of the genome by the identification of novel genes and correcting mis-annotated start codons.

sRNAs typically function by binding near the translation start site of their target mRNAs and thereby inhibit or activate translation. According to the locations of sRNA genes and their targets, sRNAs can be classified into *cis*-encoded sRNAs and *trans*-encoded sRNAs. For the *cis*-encoded sRNAs, sRNA genes overlap with their target genes. Cis-encoded regulatory RNAs are sequences overlapping with their target mRNAs that are able to change their conformation in response to an environmental cue. sRNA have been reported in the genomes of *L. biflexa*, *L. interrogans*, and *Leptospira licerasiae* (Ricaldi et al., 2012; Caimano et al., 2014; Iraola et al., 2016). An RNA thermometer, whose structure is sensitive to temperature shifts, has been shown to be responsible for the regulation of *ligA* and *ligB* expression in *L. interrogans* (Matsunaga et al., 2013). A variety of riboswitches may also operate as intracellular sensors by binding to small metabolites or ions. Cobalamin and thiamine pyrophoshate riboswitches have been previously reported in *L. interrogans* and *L. licerasiae* (Ricaldi et al., 2012; Caimano et al., 2014; Iraola et al., 2016). Binding of the effector molecule influences the secondary structure of the riboswitch part of the mRNA, which in turn affects gene expression. A previous transcriptome study of *L. interrogans* serovar Copenhageni within the mammalian host identified 11 sRNAs, which were confirmed by qRT-PCR (Caimano et al., 2014). Most of the 11 sRNAs identified in *L. interrogans* serovar Copenhageni are conserved in *L. interrogans* serovar Manilae in this study. Thus LIC2nc40, LIC2nc10 (cobalamin riboswitch), LICnc60 (RNase P), and LICnc10 (tmRNA) were also detected in our study. Other previously described sRNAs (LIC1nc80, LIC2nc20, LIC1nc11) are annotated as protein coding genes in *L. interrogans* serovar Manilae and may encode small proteins. In this study, we identified 277 and 226 putative sRNAs in *L. interrogans* serovar Manilae at 30 and 37°C, respectively, suggesting that a substantial number are novel sRNAs candidates. The relatively high number of sRNAs found in our study is likely due to differences in regards to library preparation strategies and the dRNA-seq approach used. A recent study in *B. burgdorferi*, which is one-third the size of the genome of *L. interrogans*, identified 351 putative sRNAs (Arnold et al., 2016), suggesting that spirochetes transcribe numerous noncoding RNAs which are harnessed to control transcriptional and post-transcriptional processes.

While several sRNAs have been detected in *Leptospira* spp. none had previously been experimentally validated by Northern blot. In this study, eight sRNAs were detected by Northern blotting (out of 13 tested abundant sRNAs) (**Figures 4A**, **5**).

The majority of sRNAs (>60%) are located in the intergenic regions, separated from their target genes and may act as antisense regulators on *trans*-encoded mRNAs. However, imperfect base pairing regions within their target genes makes target gene prediction challenging. We also identified cis-endoded sRNAs which are located antisense to coding regions. Notably, expression of two *cis*-encoded sRNAs may act as antisense sRNA by base pairing at the ribosome binding site (RBS) region of *lipL21* and *lipL32*, which could lead to blockage of ribosome entry and thus to the inhibition of translation of these two major and abundant lipoproteins of the cell wall. However, these sRNAs were not detectable, probably because of their low abundance in the dRNA-seq data (**Figure 4B**). Nearly half of the sRNAs (7/13) were not detectable by Northern blot. Again, this may have been due to low abundance in the dRNA-seq data. The expression levels of these sRNAs were probably below the detection limit of our non-isotopic labeling method used.

L. interrogans contains Hfq-like (LMANv2_460028) and Rho (LMANv2_80086) homologs which in many bacteria stabilizes sRNA:mRNA base-pairing interactions. The genes encoding Hfq-dependent sRNAs usually possess a typical Rho-independent transcription terminator. However, the vast majority of the

sRNAs detected in our study are not followed by a RIT and especially the number of asRNAs with RIT is marginal. Previous studies have shown that in some bacteria, the Hfq-dependent sRNAs may not contain RIT (Koo et al., 2011; Zeng and Sundin, 2014). Alternatively, Rho-dependent transcription termination may vary across the bacterial phylogeny and these termination sites can be difficult to predict using computational approaches in spirochetes.

In conclusion, we provide the first genome-wide TSS and promoter maps for the pathogen *L. interrogans*. Our approach defines TSS for most of the *L. interrogans* protein-coding genes and identifies sRNAs. Very little is known about sRNA and their potential regulatory actions in *Leptospira* spp. As more sRNAs become identified, efforts toward determining their functions will become imperative in the near future. The findings provided by this study will form the framework for future studies focused on defining the regulatory factors involved in promoting the adaptation of *L. interrogans* to the host, design of an artificial promoter system for gene studies, as well as the development of novel gene control technology, such as TALE and promoter control technology.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MP, FY, CB, CM, AZ, and JC. Performed the experiments: AZ, PH, OS, JZ, AE, and CP. Contributed reagents/materials/analysis tools: MP, CB, and CM. Wrote the paper: MP. Revised the paper: AZ, LF, PH, CP, OS, AE, and FY.

ACKNOWLEDGMENTS

This work was funded by the Institut Pasteur and FUI 14 (Fonds Unique Interministériel) COVALEPT and BPIFrance. PH was funded by Pasteur International Bioresources Network (PIBnet). CP was supported by National Science Foundation grant IIA-1159099. LF (from Dr. Ana Lucia Tabet Oller do Nascimento's lab at the Instituto Butantan, Sao Paulo, Brasil) is a doctoral recipient fellow from FAPESP. The Transcriptome and EpiGenome Platform is a member of the France Génomique consortium (ANR10-NBS-09-08).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00010/full#supplementary-material

Supplementary Table 1 | Primers used for 5′ RACE experiments.

Supplementary Table 2 | Probes used for validation of sRNA by Northern blot. For putative sRNAs, a single biotinylated probe was designed complementary to the strand that contained the candidate sRNAs.

Supplementary Table 3 | RNA-seq coverage of *L. interrogans* genes 30 and 37°C.

Supplementary Table 4 | Transcriptional Start Sites (TSS) and operons in *L. interrogans*.

Supplementary Table 5 | Shine-Dalgarno-like sequences identified near initiation codons of genes with pTSS from *L. interrogans*.

Supplementary Table 6 | Search of conserved motifs in the 80 to +1 sequences upstream of the detected pTSS at 30 and 37°C.

Supplementary Table 7 | Search of RpoD, RpoE, and RpoF binding sites in the 80 to +1 sequences upstream of the detected pTSS at 30 and 37°C.

Supplementary Table 8 | List of predicted RpoN (σ24) binding sites at 30 and 37°C.

Supplementary Table 9 | List of small RNA detected in *L. interrogans* at 30 and 37°C.

Supplementary Table 10 | Prediction of minimal free energy of sRNA secondary structure.

REFERENCES

Adams, P. P., Flores Avile, C., Popitsch, N., Bilusic, I., Schroeder, R., Lybecker, M., et al. (2016). *In vivo* expression technology and 5′ end mapping of the *Borrelia burgdorferi* transcriptome identify novel RNAs expressed during mammalian infection. *Nucleic Acids Res.* doi: 10.1093/nar/gkw1180. [Epub ahead of print].

Adler, B., Lo, M., Seemann, T., and Murray, G. L. (2011). Pathogenesis of leptospirosis: the influence of genomics. *Vet. Microbiol.* 153, 73–81. doi: 10.1016/j.vetmic.2011.02.055

Arnold, W. K., Savage, C. R., Brissette, C. A., Seshu, J., Livny, J., and Stevenson, B. (2016). RNA-Seq of *Borrelia burgdorferi* in multiple phases of growth reveals insights into the dynamics of gene expression, transcriptome architecture, and noncoding RNAs. *PLoS ONE* 11:e0164165. doi: 10.1371/journal.pone.0164165

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., et al. (2009). MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 37, W202–W208. doi: 10.1093/nar/gkp335

Ballard, S. A., Segers, R. P., Bleumink-Pluym, N., Fyfe, J., Faine, S., and Adler, B. (1993). Molecular analysis of the hsp (groE) operon of *Leptospira interrogans* serovar copenhageni. *Mol. Microbiol.* 8, 739–751. doi: 10.1111/j.1365-2958.1993.tb01617.x

Barrios, H., Valderrama, B., and Morett, E. (1999). Compilation and analysis of sigma 54-dependent promoter sequences. *Nucleic Acids Res.* 27, 4305–4313. doi: 10.1093/nar/27.22.4305

Bischler, T., Tan, H. S., Nieselt, K., and Sharma, C. M. (2015). Differential RNA-seq (dRNA-seq) for annotation of transcriptional start sites and small RNAs in *Helicobacter pylori*. *Methods* 86, 89–101. doi: 10.1016/j.ymeth.2015.06.012

Bullard, J. H., Purdom, E., Hansen, K. D., and Dudoit, S. (2010). Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC Bioinformatics* 11:94. doi: 10.1186/1471-2105-11-94

Caimano, M. J., Sivasankaran, S. K., Allard, A., Hurley, D., Hokamp, K., Grassmann, A. A., et al. (2014). A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar Copenhageni. *PLoS Pathog.* 10:e1004004. doi: 10.1371/journal.ppat.1004004

Cortes, T., Schubert, O. T., Rose, G., Arnvig, K. B., Comas, I., Aebersold, R., et al. (2013). Genome-wide mapping of transcriptional start sites defines an extensive leaderless transcriptome in *Mycobacterium tuberculosis*. *Cell Rep.* 5, 1121–1131. doi: 10.1016/j.celrep.2013.10.031

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

Criscuolo, A., and Brisse, S. (2013). AlienTrimmer: a tool to quickly and accurately trim off multiple short contaminant sequences from high-throughput sequencing reads. *Genomics* 102, 500–506. doi: 10.1016/j.ygeno.2013.07.011

Cuñé, J., Cullen, P., Mazon, G., Campoy, S., Adler, B., and Barbe, J. (2005). The *Leptospira interrogans* lexA gene is not autoregulated. *J. Bacteriol.* 187, 5841–5845. doi: 10.1128/JB.187.16.5841-5845.2005

de Groot, A., Roche, D., Fernandez, B., Ludanyi, M., Cruveiller, S., Pignol, D., et al. (2014). RNA sequencing and proteogenomics reveal the importance of leaderless mRNAs in the radiation-tolerant bacterium *Deinococcus deserti*. *Genome Biol. Evol.* 6, 932–948. doi: 10.1093/gbe/evu069

Dugar, G., Herbig, A., Förstner, K. U., Heidrich, N., Reinhardt, R., Nieselt, K., et al. (2013). High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet.* 9:e1003495. doi: 10.1371/journal.pgen.1003495

Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30, 207–210. doi: 10.1093/nar/30.1.207

Ellinghausen, H. C. Jr., and McCullough, W. G. (1965). Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.* 26, 45–51.

Fouts, D. E., Matthias, M. A., Adhikarla, H., Adler, B., Berg, D. E., Bulach, D., et al. (2016). What makes a bacterial species pathogenic?: Comparative genomic analysis of the genus *Leptospira*. *PLoS Negl. Trop. Dis.* 10:e0004403. doi: 10.1371/journal.pntd.0004403

Grant, C. E., Bailey, T. L., and Noble, W. S. (2011). FIMO: scanning for occurrences of a given motif. *Bioinformatics* 27, 1017–1018. doi: 10.1093/bioinformatics/btr064

Gupta, S., Stamatoyannopoulos, J. A., Bailey, T. L., and Noble, W. S. (2007). Quantifying similarity between motifs. *Genome Biol.* 8:R24. doi: 10.1186/gb-2007-8-2-r24

Hertz, G. Z., and Stormo, G. D. (1999). Identifying, D. N. A., and protein patterns with statistically significant alignments of multiple sequences. *Bioinformatics* 15, 563–577. doi: 10.1093/bioinformatics/15.7.563

Hu, W. L., Pappas, C. J., Zhang, J. J., Yang, Y. Y., Yan, J., Picardeau, M., et al. (2017). The EbpA-RpoN regulatory pathway of the pathogen *Leptospira interrogans* is essential for survival in the environment. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.02377-16. [Epub ahead of print].

Iraola, G., Spangenberg, L., Lopes Bastos, B., Graña, M., Vasconcelos, L., Almeida, Á., et al. (2016). Transcriptome sequencing reveals wide expression reprogramming of basal and unknown genes in *Leptospira biflexa* biofilms. *mSphere* 1, e00042–e00016. doi: 10.1128/mSphere.00042-16

Kazmierczak, M. J., Wiedmann, M., and Boor, K. J. (2005). Microbiology and molecular biology reviews. Alternative sigma factors and their roles in *Bacterial Virulence. Microb. Mol. Biol. Rev.* 69, 527–543. doi: 10.1128/MMBR.69.4.527-543.2005

Kiliç, S., White, E. R., Sagitova, D. M., Cornish, J. P., and Erill, I. (2014). CollecTF: a database of experimentally validated transcription factor-binding sites in Bacteria. *Nucleic Acids Res.* 42, D156–D160. doi: 10.1093/nar/gkt1123

Ko, A. I., Goarant, C., and Picardeau, M. (2009). Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* 7, 736–747. doi: 10.1038/nrmicro2208

Koo, J. T., Alleyne, T. M., Schiano, C. A., Jafari, N., and Lathem, W. W. (2011). Global discovery of small RNAs in Yersinia pseudotuberculosis identifies Yersinia-specific small, noncoding RNAs required for virulence. *Proc. Natl. Acad. Sci. U.S.A.* 108, E709–E717. doi: 10.1073/pnas.1101655108

Kröger, C., Dillon, S. C., Cameron, A. D., Papenfort, K., Sivasankaran, S. K., Hokamp, K., et al. (2012). The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1277–1286. doi: 10.1073/pnas.1201061109

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Liao, Y., Huang, L., Wang, B., Zhou, F., and Pan, L. (2015). The global transcriptional landscape of *Bacillus amyloliquefaciens* XH7 and high-throughput screening of strong promoters based on RNA-seq data. *Gene* 571, 252–262. doi: 10.1016/j.gene.2015.06.066

Lo, M., Bulach, D. M., Powell, D. R., Haake, D. A., Matsunaga, J., Paustian, M. L., et al. (2006). Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. *Infect. Immun.* 74, 848–859. doi: 10.1128/IAI.00755-06

Lo, M., Murray, G. L., Khoo, C. A., Haake, D. A., Zuerner, R. L., and Adler, B. (2010). Transcriptional response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog. *Infect. Immun.* 78, 4850–4859. doi: 10.1128/IAI.00435-10

Malmström, J., Beck, M., Schmidt, A., Lange, V., Deutsch, E. W., and Aebersold, R. (2009). Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature* 460, 762–765. doi: 10.1038/nature08184

Markham, N. R., and Zuker, M. (2008). UNAFold: software for nucleic acid folding and hybridization. *Methods Mol. Biol.* 453, 3–31. doi: 10.1007/978-1-60327-429-6_1

Matsunaga, J., Lo, M., Bulach, D. M., Zuerner, R. L., Adler, B., and Haake, D. A. (2007). Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. *Infect. Immun.* 75, 2864–2874. doi: 10.1128/IAI.01619-06

Matsunaga, J., Schlax, P. J., and Haake, D. A. (2013). Role for cis-acting RNA sequences in the temperature-dependent expression of the multiadhesive lig proteins in *Leptospira interrogans*. *J. Bacteriol.* 195, 5092–5101. doi: 10.1128/JB.00663-13

McClure, R., Balasubramanian, D., Sun, Y., Bobrovskyy, M., Sumby, P., Genco, C. A., et al. (2013). Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res.* 41:e140. doi: 10.1093/nar/gkt444

Morero, N. R., Botti, H., Nitta, K. R., Carrión, F., Obal, G., Picardeau, M., et al. (2014). HemR is an OmpR/PhoB-like response regulator from Leptospira, which simultaneously effects transcriptional activation and repression of key haem metabolism genes. *Mol. Microbiol.* 94, 340–352. doi: 10.1111/mmi.12763

Naville, M., Ghuillot-Gaudeffroy, A., Marchais, A., and Gautheret, D. (2011). ARNold: a web tool for the prediction of Rho-independent transcription terminators. *RNA Biol.* 8, 11–13. doi: 10.4161/rna.8.1.13346

Nawrocki, E. P., Burge, S. W., Bateman, A., Daub, J., Eberhardt, R. Y., Eddy, S. R., et al. (2015). Rfam 12.0: updates to the RNA families database. *Nucleic Acids Res.* 43, D130–D137. doi: 10.1093/nar/gku1063

Noguchi, H., Taniguchi, T., and Itoh, T. (2008). MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes. *DNA Res.* 15, 387–396. doi: 10.1093/dnares/dsn027

Oliphant, T. E. (2007). Python for scientific computing. *Comput. Sci. Eng.* 9, 10–20. doi: 10.1109/MCSE.2007.58

Pachkov, M., Balwierz, P. J., Arnold, P., Ozonov, E., and van Nimwegen, E. (2013). SwissRegulon, a database of genome-wide annotations of regulatory sites: recent updates. *Nucleic Acids Res.* 41, D214–D220. doi: 10.1093/nar/gks1145

Pappas, C. J., and Picardeau, M. (2015). Control of gene expression in *Leptospira* spp. by Transcription Activator-Like Effectors (TALEs) demonstrates a potential role for LigA and LigB in virulence in *L.* interrogans. *Appl. Environ. Microbiol.* 81, 7888–7892. doi: 10.1128/AEM.02202-15

Patarakul, K., Lo, M., and Adler, B. (2010). Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. *BMC Microbiol.* 10:31. doi: 10.1186/1471-2180-10-31

Picardeau, M. (2015). "Genomics, proteomics, and genetics of leptospira," in *Leptospira and Leptospirosis,* ed B. Adler (Heidelberg: Springer-Verlag Berlin), 43–63.

Qin, J. H., Sheng, Y. Y., Zhang, Z. M., Shi, Y. Z., He, P., Hu, B. Y., et al. (2006). Genome-wide transcriptional analysis of temperature shift in L. interrogans serovar lai strain 5660. *BMC Microbiol.* 6:51. doi: 10.1186/1471-2180-6-51

Quinlan, A. R. (2014). BEDTools: The Swiss-Army tool for genome feature analysis. *Curr. Protoc. Bioinform.* 47, 11.12.1–11.12.34. doi: 10.1002/0471250953.bi1112s47

Ren, S. X., Fu, G., Jiang, X. G., Zeng, R., Miao, Y. G., Xu, H., et al. (2003). Unique and physiological and pathogenic features of *Leptospira interrogans* revealed by whole genome sequencing. *Nature* 422, 888–893. doi: 10.1038/nature01597

Ricaldi, J. N., Fouts, D. E., Selengut, J. D., Harkins, D. M., Patra, K. P., Moreno, A., et al. (2012). Whole genome analysis of *Leptospira licerasiae* provides insight into Leptospiral evolution and pathogenicity. *PLoS Negl. Trop. Dis.* 6:e1853. doi: 10.1371/journal.pntd.0001853

Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., et al. (2011). Integrative genomics viewer. *Nat. Biotechnol.* 29, 24–26. doi: 10.1038/nbt.1754

Sahr, T., Rusniok, C., Dervins-Ravault, D., Sismeiro, O., Coppee, J. Y., and Buchrieser, C. (2012). Deep sequencing defines the transcriptional map of *L. pneumophila* and identifies growth phase-dependent regulated ncRNAs implicated in virulence. *RNA Biol.* 9, 503–519. doi: 10.4161/rna.20270

Schlüter, J. P., Reinkensmeier, J., Barnett, M. J., Lang, C., Krol, E., Giegerich, R., et al. (2013). Global mapping of transcription start sites and promoter motifs in the symbiotic α-proteobacterium *Sinorhizobium meliloti* 1021. *BMC Genomics* 14:156. doi: 10.1186/1471-2164-14-156

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27, 863–864. doi: 10.1093/bioinformatics/btr026

Tatusov, R. L. (1997). A genomic perspective on protein families. *Science* 278, 631–637. doi: 10.1126/science.278.5338.631

Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A., et al. (2013). MicroScope–an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* 41, D636–D647. doi: 10.1093/nar/gks1194

Voigt, K., Sharma, C. M., Mitschke, J., Lambrecht, S. J., Voß, B., Hess, W. R., et al. (2014). Comparative transcriptomics of two environmentally relevant cyanobacteria reveals unexpected transcriptome diversity. *ISME J.* 8, 2056–2068. doi: 10.1038/ismej.2014.57

Wurtzel, O., Sapra, R., Chen, F., Zhu, Y., Simmons, B. A., and Sorek, R. (2010). A single-base resolution map of an archaeal transcriptome. *Genome Res.* 20, 133–141. doi: 10.1101/gr.100396.109

Wurtzel, O., Yoder-Himes, D. R., Han, K., Dandekar, A. A., Edelheit, S., Greenberg, E. P., et al. (2012). The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. *PLoS Pathog.* 8:e1002945. doi: 10.1371/journal.ppat.1002945

Xue, F., Dong, H., Wu, J., Wu, Z., Hu, W., Sun, A., et al. (2010). Transcriptional responses of *Leptospira interrogans* to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. *PLoS Negl. Trop. Dis.* 4:e857. doi: 10.1371/journal.pntd.0000857

Zeng, Q., and Sundin, G. W. (2014). Genome-wide identification of Hfq-regulated small RNAs in the fire blight pathogen *Erwinia amylovora* discovered small RNAs with virulence regulatory function. *BMC Genomics* 15:14. doi: 10.1186/1471-2164-15-414

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 27 March 2018
doi: 10.3389/fcimb.2018.00092

Leptospira interrogans Secreted Proteases Degrade Extracellular Matrix and Plasma Proteins From the Host

*Ludmila B. da Silva[1], Milene C. Menezes[2], Eduardo S. Kitano[2], Ana K. Oliveira[3], Afonso G. Abreu[4,5], Gisele O. Souza[6], Marcos B. Heinemann[6], Lourdes Isaac[7], Tatiana R. Fraga[7], Solange M. T. Serrano[2] and Angela S. Barbosa[1]**

[1] Laboratory of Bacteriology, Butantan Institute, São Paulo, Brazil, [2] Special Laboratory of Applied Toxinology, Center of Toxins, Immune-Response and Cell Signaling, Butantan Institute, São Paulo, Brazil, [3] Brazilian Biosciences National Laboratory, Brazilian Center for Research in Energy and Materials, Campinas, São Paulo, Brazil, [4] Postgraduation Program in Parasitic Biology, CEUMA University, São Luís, Brazil, [5] Postgraduation Program in Health Sciences, Federal University of Maranhão, São Luís, Brazil, [6] Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil, [7] Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Azad Eshghi,
UVic Genome BC Protein Centre, Canada

Reviewed by:
Christopher J. Pappas,
Manhattanville College, United States
J. Christopher Fenno,
University of Michigan, United States
Peter Kraiczy,
Goethe University Frankfurt, Germany

***Correspondence:**
Angela S. Barbosa
angela.barbosa@butantan.gov.br

Received: *27 October 2017*
Accepted: *07 March 2018*
Published: *27 March 2018*

Citation:
da Silva LB, Menezes MC, Kitano ES, Oliveira AK, Abreu AG, Souza GO, Heinemann MB, Isaac L, Fraga TR, Serrano SMT and Barbosa AS (2018) Leptospira interrogans Secreted Proteases Degrade Extracellular Matrix and Plasma Proteins From the Host. Front. Cell. Infect. Microbiol. 8:92. doi: 10.3389/fcimb.2018.00092

Leptospires are highly motile spirochetes equipped with strategies for efficient invasion and dissemination within the host. Our group previously demonstrated that pathogenic leptospires secrete proteases capable of cleaving and inactivating key molecules of the complement system, allowing these bacteria to circumvent host's innate immune defense mechanisms. Given the successful dissemination of leptospires during infection, we wondered if such proteases would target a broader range of host molecules. In the present study, the proteolytic activity of secreted leptospiral proteases against a panel of extracellular matrix (ECM) and plasma proteins was assessed. The culture supernatant of the virulent *L. interrogans* serovar Kennewicki strain Fromm (LPF) degraded human fibrinogen, plasma fibronectin, gelatin, and the proteoglycans decorin, biglycan, and lumican. Interestingly, human plasminogen was not cleaved by proteases present in the supernatants. Proteolytic activity was inhibited by 1,10-phenanthroline, suggesting the participation of metalloproteases. Moreover, production of proteases might be an important virulence determinant since culture-attenuated or saprophytic *Leptospira* did not display proteolytic activity against ECM or plasma components. Exoproteomic analysis allowed the identification of three metalloproteases that could be involved in the degradation of host components. The ability to cleave conjunctive tissue molecules and coagulation cascade proteins may certainly contribute to invasion and tissue destruction observed upon infection with *Leptospira*.

Keywords: *Leptospira*, secreted proteins, extracellular matrix, plasma proteins, host invasion

INTRODUCTION

Leptospira are long, thin, spiral-shaped, and highly motile Gram-negative bacteria. These spirochetes can be either non-pathogenic free-living organisms or pathogenic, having the potential to cause disease in animals and humans. During infection, leptospires invade multiple organs and tissues, and damage the endothelial linings of the small blood vessels. Severe cases of leptospirosis are characterized by multiple symptoms that may include vascular injury, thrombocytopenia, jaundice, kidney failure, pulmonary hemorrhage, and ocular manifestations such as uveitis and conjunctival congestion (Levett, 2001).

Pathogenic leptospires efficiently spread and propagate in susceptible hosts. They are equipped with strategies to modulate the surrounding microenvironment in the host, including mechanisms to circumvent host's immune responses (Meri et al., 2005; Barbosa et al., 2009). The secretion of proteases that inactivate essential host proteins is an important tool used by diverse microorganisms during the colonization process. *Leptospira* is no exception to this phenomenon, since pathogenic strains have been shown to secrete proteases capable of degrading several proteins of the complement cascade, contributing to serum resistance (Fraga et al., 2014; Amamura et al., 2017). Many bacterial proteolytic enzymes can be considered virulence factors. By hydrolyzing diverse proteinaceous substrates of the host, bacterial proteases play a crucial role in colonization and spreading, allowing evasion of innate immune responses and contributing to disruption of tissue integrity.

The knowledge on the mechanisms underlying tissue damage during leptospirosis is still limited. Like other bacteria, leptospires cross epithelial and endothelial host barriers to get access to target organs. Adhesion to and degradation of the extracellular matrix (ECM), notably of basement membranes, are certainly required for invasion. To date, few studies have been conducted to experimentally identify and characterize leptospiral proteases. It has been shown that a number of *Leptospira* serovars produce multiple gelatinases ranging from 32 to 240 kDa (Madathiparambil et al., 2011). One of them, named ColA, was further characterized and was shown to hydrolyze different types of collagen. A *colA* mutant strain displayed attenuated transcytosis through human embryonic kidney cell lineage HEK293 and human umbilical vein endothelial cell (HUVEC) monolayers, and reduced virulence in the hamster model of infection. A reduced number of bacteria in organs of animals infected with the mutant strain was also observed (Kassegne et al., 2014). Leptallo I, a protease that belongs to the M23 family, displays proteolytic activity against elastin. Leptallo I was shown to be secreted to the culture medium during leptospiral growth, and IgG antibodies recognizing the protein could be detected in the sera of patients with laboratory-confirmed leptospirosis (Hashimoto et al., 2013).

In a previous work, we reported the secretion of proteases by pathogenic *Leptospira* as a novel complement evasion mechanism displayed by these spirochetes (Fraga et al., 2014). Given the rapid and successful dissemination of leptospires during infection, we can assume that such proteases target a broader range of host molecules. As such, the purpose of this work was to evaluate the proteolytic activity of secreted leptospiral proteases against a panel of ECM and plasma proteins.

MATERIALS AND METHODS

Proteins, Antibodies, and Plasma

Fibrinogen, plasminogen, thrombin and fibronectin from human plasma, laminin from basement membrane Engelbreth-Holm-Swarm (EHS) murine sarcoma, and decorin from bovine articular cartilage were purchased from Sigma-Aldrich. Gelatin and Matrigel from EHS murine sarcoma were purchased from Difco and BD Biosciences, respectively, and the recombinant proteoglycans lumican (human), and biglycan (human) were purchased from R&D Systems. Rabbit anti-human fibrinogen was from Sigma-Aldrich. Depletion of albumin from human plasma was performed according to Subramanian (1984).

Leptospira Strains

The *Leptospira* strains used were *L. biflexa* serovar Patoc strain Patoc I, *L. biflexa* serovar Andamana strain CH11, *L. interrogans* serovar Kennewicki strain Fromm (LPF), *L. interrogans* serovar Copenhageni strain 10A, *L. interrogans* serovar Pomona strain Pomona, *L. kirshneri* serovar Cynopteri strain 3522 C, and *L. noguchi* serovar Panama CZ 214. Virulence of LPF was maintained by iterative passages in hamsters. Infected animals become acutely ill and present symptoms such as jaundice, uveitis, light sensitivity, prostration, loss of appetite. They usually die on day 5 post-infection. Bacteria were cultured for 7 days in modified Ellinghausen-McCullough-Johnson-Harris (EMJH) at 29°C under aerobic conditions as previously described (Barbosa et al., 2010). The leptospires were then washed, counted by dark-field microscopy, and 1×10^9 bacteria resuspended in PBS (pH 7.4) were allowed to secrete proteins for 4 h at 37°C, thus simulating responses to temperature upshift observed during infection of mammalian hosts (Fraga et al., 2014). Protein concentration was determined using the BCA Kit (Pierce).

Degradation of Plasma and ECM Molecules

Fibrinogen (15 μg), plasminogen (5 μg), fibronectin (5 μg), thrombin (1 μg), laminin (5 μg), matrigel (5 μg), decorin (2 μg), biglycan (2 μg), and lumican (2 μg) were incubated with culture supernatants from different *Leptospira* strains (0.5 μg) at 37°C for the indicated time points. A sample of each substrate was also incubated without culture supernatants for 18 h under identical conditions. Reactions were stopped by adding Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and subjected to SDS-PAGE. Polyacrylamide gels were subsequently stained. Degradation of fibrinogen in plasma was assessed as follows. Albumin-depleted human plasma (15 μg) was incubated with LPF supernatant (0.01–0.5 μg) for 2 h at 37°C and then submitted to SDS-PAGE followed by electroblotting onto nitrocellulose membrane. Cleavage products were detected using rabbit anti-human fibrinogen antibodies, followed by peroxidase-conjugated anti-rabbit antibodies. Positive signals were detected

by enhanced chemiluminescence (West Pico, Pierce). To assess the class of proteases involved in the degradation of ECM and plasma proteins, leptospiral supernatants were preincubated with inhibitors of serine (5 mmol/L phenylmethylsulfonyl fluoride), metallo- (5 mmol/L 1,10-phenanthroline), cysteine (28 μmol/L E-64) or aspartyl (5 μmol/L pepstatin) proteases for 30 min before the addition of the substrate.

Gelatin Zymography

To assess gelatinolytic enzymatic activity in *Leptospira* culture supernatants zymography using gelatin was performed. Gelatin (1 mg/mL) was copolymerized with 12% w/v acrylamide, 0.3% bisacrylamide and 0.375 M Tris-HCl (pH 8.8). Samples (0.5 μg of proteins from culture supernatants) were mixed with non-reducing SDS-PAGE sample buffer (40 mM Tris HCl pH 6.8, 1% SDS, 2% glycerol and 0.01% bromophenol blue) and applied to gels. After electrophoresis, the gels were incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris-HCl, pH 7.4, containing 2.5% Triton X-100. Excess Triton X-100 was removed upon washes with deionized water and then the gels were incubated in a buffer containing 0.05 M Tris–HCl, pH 8.0, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.02% CHAPS at 37°C for 12 h. The gels were stained with Coomassie blue and destained. Gelatin digestion was identified as clear zones of lysis against a blue background.

Mass Spectrometry and Protein Identification

L. interrogans (LPF) and *L. biflexa* (Patoc I) (10^9 bacteria) cultured in EMJH medium at 29°C were pelleted by centrifugation at 3,200 × g for 15 min, and washed twice with PBS, pH 7.4. The bacteria were resuspended in PBS and incubated at 37°C for 4 h to allow secretion of proteins, and centrifuged at 3,200 × g for 10 min. Supernatants were collected, passed through a 0.22 μm filter (Merck Millipore), and concentrated using Amicon Ultra centrifugal filters (3,000 MWCO; Merck Millipore).

For the identification of *Leptospira* secreted proteins, 30 μg of proteins derived from LPF and Patoc I culture supernatants (three biological replicates) were processed by the Filter Aided Sample Preparation (FASP) procedure (Wiśniewski et al., 2009), using 10 kDa Microcon filtration devices (Millipore). The resulting protein mixture was diluted to 0.06 mL of 0.05 M NH_4HCO_3 and trypsin (Sigma) was added at a 1:50 enzyme-to-substrate ratio, and submitted to incubation at 37°C for 18 h. Peptide samples were desalted using StageTips C18 according to Rappsilber et al. (2007).

Each peptide mixture (5 μL) was injected into a 5 cm Jupiter® C-18 trap column packed in-house (Phenomenex; 100 μm I.D. × 360 μm O.D.) using a EASY nanoLCII system (Thermo Fisher Scientific) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Chromatographic separation of tryptic peptides was performed on a 10-cm long column (75 μm I.D. × 360 μm O.D.) packed in-house with 5 μm Aqua® C-18 beads (Phenomenex). Peptides eluted with a linear gradient of 5–35% acetonitrile in 0.1% formic acid (solution B) at 200 nL/min in 75 min, 35–80% B in 15 min, 80% B for 7 min, back to 5% in 1 min and 5% B for 22 min. Spray voltage was set to 2.4 kV and the mass spectrometer was operated in data dependent mode, in which one full MS scan was acquired in the *m*/*z* range of 300–1,700 followed by MS/MS acquisition using collision induced dissociation of the twelve most intense ions from the MS scan. MS spectra were acquired in the Orbitrap analyzer at 60,000 resolutions (at 400 m/z) whereas the MS/MS scans were acquired in the linear ion trap. Isolation window, activation time and normalized collision energy were set to, respectively, 3 m/z, 10 ms and 35%. A dynamic peak exclusion was applied to avoid the same *m*/*z* of being selected for the next 60 s. Three technical replicates were performed.

LTQ-Orbitrap Velos raw data were searched using the Andromeda algorithm (Cox et al., 2011) at the MaxQuant environment (Cox and Mann, 2008; version 1.5.0.0), using a target database restricted to the genus *Leptospira* (UniProt database containing 366,195 protein sequences, downloaded on Nov 30, 2015) to which a set of reverse sequences were added (decoy dataset), with a parent and fragment mass tolerance of 10 ppm and 0.5 Da, respectively. Carbamidomethylation was set as fixed modification, and oxidation of methionine, protein N-acetylation and deamidation of asparagine or glutamine were specified as variable modifications. Two missed cleavages were allowed and the minimal length required for a peptide was seven amino acids. One unique peptide was required for protein identifications. The false discovery rate (FDR) at peptide and protein level was adjusted to 1%. The output of the search was processed using Perseus (Tyanova et al., 2016; version 1.5.0.15). Contaminants, reverse decoy proteins, and proteins identified only by a modification site were removed from search. Only proteins identified in two of three biological replicate data sets were accepted.

RESULTS

Degradation of ECM and Plasma Proteins by *L. interrogans*

Degradation of structural and soluble host molecules contributes to bacterial dissemination facilitating invasion and colonization of target organs. In this work, we evaluated the proteolytic activity of leptospiral secreted proteases against ECM and plasma proteins. Secreted proteins were obtained upon incubation of leptospires at 37°C in PBS because it has been previously shown that proteolytic activity against host's complement molecules is clearly observed under these conditions (Fraga et al., 2014).

Basement membranes, predominantly composed of laminin and collagen IV, are specialized extracellular matrices that separate the epithelium and endothelium from underlying connective tissue. Since degradation of basement membrane components is crucial for invasion, we first assessed proteolytic activity of *Leptospira* culture supernatants against laminin and matrigel. Under our experimental conditions, laminin and matrigel were not degraded by proteins from leptospiral supernatants (**Figures 1A,B**).

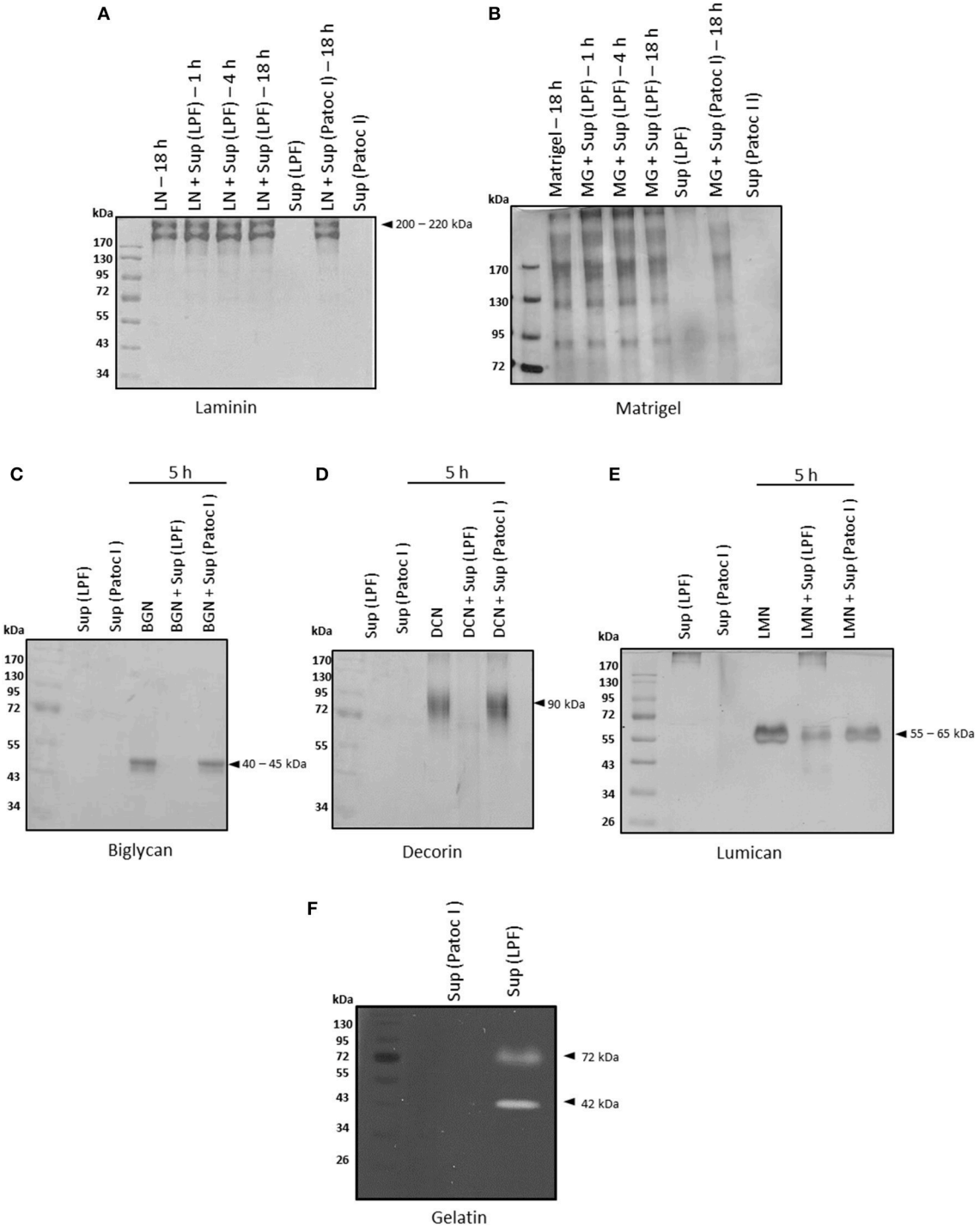


FIGURE 1 | Degradation of ECM proteins by *Leptospira interrogans* secreted proteins. **(A)** Laminin (5 μg), **(B)** matrigel (5 μg), **(C)** biglycan (2 μg), **(D)** decorin (2 μg), and **(E)** lumican (2 μg) were incubated with supernatants of pathogenic *Leptospira interrogans* serovar Kennewicki strain Fromm (LPF) or saprophytic *Leptospira biflexa* serovar Patoc strain Patoc I (0.5 μg of total secreted proteins) at 37°C for the indicated time points. Cleavage products were evaluated by SDS-PAGE under reducing conditions. Gels were silver stained. **(F)** Proteins present in both supernatants (0.5 μg of total secreted proteins) were submitted to zymography on a 12% SDS-polyacrylamide gel copolymerized with gelatin and Coomassie Brilliant Blue stained. LN, laminin; MG, matrigel; BGN, biglycan; DCN, decorin; LMN, lumican; Sup (LPF), supernatant of *Leptospira interrogans* serovar Kennewicki strain Fromm; Sup (Patoc), supernatant of *Leptospira biflexa* serovar Patoc strain Patoc I.

Proteoglycans, heavily glycosylated proteins, are also major components of extracellular matrices. They fill spaces in the ECM by bridging different macromolecules and provide a highly hydrated gel-like microenvironment (revised in Chagnot et al., 2012). Degradation of small leucine-rich proteoglycans (SLRP) by proteases present in leptospiral culture supernatants was also assessed. At an enzyme/substrate ratio of 1: 4, i.e., 0.5 μg of supernatant: 2 μg of proteoglycan, decorin and biglycan were fully degraded after incubation with the virulent strain LPF supernatant whereas lumican was partially degraded (**Figures 1C–E**). At an enzyme/substrate ratio of 1: 10, proteoglycan hydrolysis was much less pronounced (data not shown).

In addition, proteases of approximately 72 and 42 kDa with gelatinolytic activity were detected in the presence of the LPF supernatant, as revealed by gel-zymography (**Figure 1F**). Regardless of the substrate used, no proteolytic activity was observed upon incubation with the saprophytic strain Patoc I supernatant (**Figure 1**).

Blood coagulation proteins such as fibrinogen, plasminogen, and thrombin, as well as plasma fibronectin, were also tested as substrates for leptospiral proteases. Plasma fibronectin and fibrinogen were time-dependently degraded by LPF proteases whereas no degradation occurred in the presence of Patoc I supernatant, even after 18 h of incubation (**Figures 2A,B**). Fibronectin fragments ranging from 25 to 170 kDa were generated upon a prolonged incubation period (**Figure 2A**). Smaller fibrinogen fragments of 25–45 kDa were also produced as a consequence of both α- and β-chain degradation (**Figure 2B**). An enzyme/substrate ratio of 1: 30, i.e., 0.5 μg of supernatant: 15 μg of purified fibrinogen, seems to be the minimum required ratio for complete degradation of both α and β chains (**Supplementary Figure 1**), and this condition was then chosen for subsequent assays. Proteolytic activity using albumin-depleted human plasma further confirmed fibrinogen degradation by proteinases present in the LPF supernatant (**Supplementary Figure 1**). Reduced amounts of leptospiral proteases can degrade fibrinogen. By contrast, plasminogen and thrombin were not susceptible to leptospiral proteases, as depicted in **Figures 2C,D**.

Metalloproteases Secreted by *L. interrogans* Degrade Proteoglycans, Plasma Fibronectin and Fibrinogen

To assess the class(es) of proteases involved in the degradation of host molecules, LPF supernatant was pretreated with inhibitors of serine, metallo-, cysteine, or aspartyl proteases before the addition of each substrate. In all cases, only 1,10-phenanthroline could fully inhibit the proteolytic activities, strongly suggesting the involvement of metalloproteases in this process (**Figure 3**, lane 4).

Proteolytic Activity Against Host Molecules Correlates With *L. interrogans* Virulence

To assess the proteolytic potential of additional *Leptospira* strains, we used a panel of seven *Leptospira* strains including one virulent (*L. interrogans* serovar Kennewicki strain Fromm, also called "LPF"), two saprophytes (*L. biflexa* serovar Patoc strain Patoc I and *L. biflexa* serovar Andamana strain CH11), and four culture-attenuated (*L. interrogans* serovar Copenhageni strain 10A, *L. interrogans* serovar Pomona strain Pomona, *L. kirshneri* serovar Cynopteri strain 3522 C and *L. noguchi* serovar Panama CZ 214). Strains LPF and Patoc I were used in the previous assays presented in **Figures 1–3**. Interestingly, proteolytic activity was only observed in the presence of the virulent strain LPF supernatant, thus indicating that production of proteases might be an important virulence determinant (**Figure 4**). Culture-attenuated or saprophytic *Leptospira* strains did not display a significant proteolytic activity against fibronectin, decorin or fibrinogen (**Figure 4**). Culture supernatants from the seven *Leptospira* strains used in this study were the same as those used in Fraga et al. (2014).

Identification of Secreted Proteins From LPF and Patoc I Strains

All secreted proteins identified in the LPF and Patoc I strains are shown in **Supplementary Table 1**. A total of 236 and 161 unique proteins were identified, respectively, in the LPF and in the Patoc I exoproteomes (**Supplementary Tables 2**, **3**). Fifty proteins were detected in both strains (**Supplementary Table 4**). A search aiming to find proteases unique to the LPF exoproteome allowed identification of six peptidases, out of which three are metalloproteases (**Table 1**). One of them, a 52 kDa protein belonging to the M43 family, harbors a pappalysin-1 domain and contains a HEXXHXXGXXH zinc-dependent active site. Human pappalysin-1 is described as a secreted metalloprotease which cleaves insulin-like growth factor binding proteins (Oxvig, 2015). This protein also plays a role in bone formation, inflammation, wound healing and female fertility. A role for proteins containing pappalysin-1 domains remains to be exploited in bacteria. Another metalloprotease identified was TldD, a 49.9 kDa protease containing the conserved HEXXH zinc-binding motif. TldD is quite conserved among prokaryotes, and plays a role in degrading unfolded proteins, as well as in the activation and degradation of natural products such as the peptide antibiotic microcin B17, peptide-derived cofactors or toxin-antitoxin modules (Ghilarov et al., 2017). The third metalloproatese identified was the 26.6 kDa methionine aminopeptidase (MAP) known to catalyze the hydrolytic cleavage of the N-terminal methionine from newly synthesized polypeptides. Apparently, neither of the three aforementioned proteases seems to be involved in gelatin hydrolysis since according to our zymography data gelatinolytic activity was associated with proteases of 72 and 42 kDa (**Figure 1F**). Not infrequently, proteases are synthesized in very small quantities despite being highly efficient in hydrolyzing substrates, what may explain the non-detection of proteases presenting molecular masses of 72 and 42 kDa in our proteomic analysis.

LPF and Patoc I secreted proteins were assigned to clusters of orthologous groups (COGs). Under molecular function the proteins were classified into the following categories: catalytic activity, binding, structural molecule activity, antioxidant activity, transporter activity, and nutrient reservoir activity.

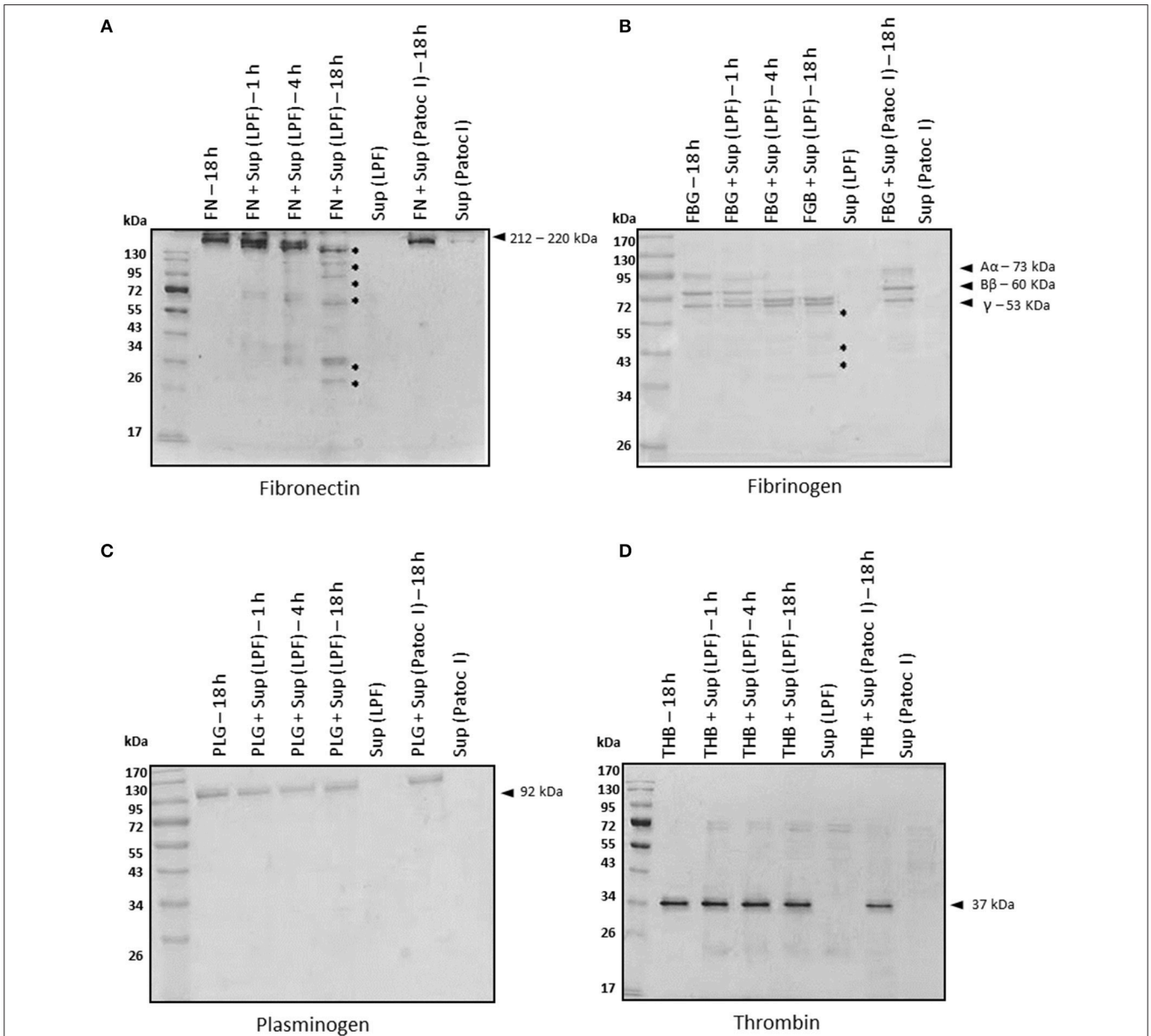


FIGURE 2 | Degradation of plasma proteins by *Leptospira interrogans* secreted proteases**. (A)** Plasma fibronectin (5 μg), **(B)** fibrinogen (15 μg), **(C)** plasminogen (5 μg), and **(D)** thrombin (1 μg) were incubated with supernatants of pathogenic *Leptospira interrogans* serovar Kennewicki strain Fromm or saprophytic *Leptospira biflexa* serovar Patoc strain Patoc I (0.5 μg of total secreted proteins) at 37°C for the indicated time points. Cleavage products were evaluated by SDS- polyacrylamide gel under reducing conditions. Gels were silver stained. FN, plasma fibronectin; FBG, fibrinogen; PLG, plasminogen; THB, thrombin; Sup (LPF), supernatant of *Leptospira interrogans* serovar Kennewicki strain Fromm; Sup (Patoc), supernatant of *Leptospira biflexa* serovar Patoc strain Patoc I.

Proteins involved in catalytic activity and binding were the most represented in both LPF and Patoc I strains (**Figure 5**).

Using the MoonProt database (Mani et al., 2015), 25 proteins presumed to display moonlighting functions were detected (**Supplementary Table 5**). These multifunctional proteins play crucial roles in physiological processes, but have been shown to contribute to infection by many pathogens (Henderson, 2017). Housekeeping enzymes, including proteins from the glycolytic pathway, and chaperones are among the moonlighting proteins found in the exoproteomes.

DISCUSSION

Proteolytic activity targeting host molecules is a key strategy to facilitate the infection process of pathogens. Successful infection is not only achieved by the ability to circumvent host's innate immune defenses, but also by additional mechanisms that may aid bacteria to reach target organs. In this context, secreted proteases are of major importance for the infectious process. They are produced by pathogens including both Gram-positive and Gram-negative bacteria, viruses, and

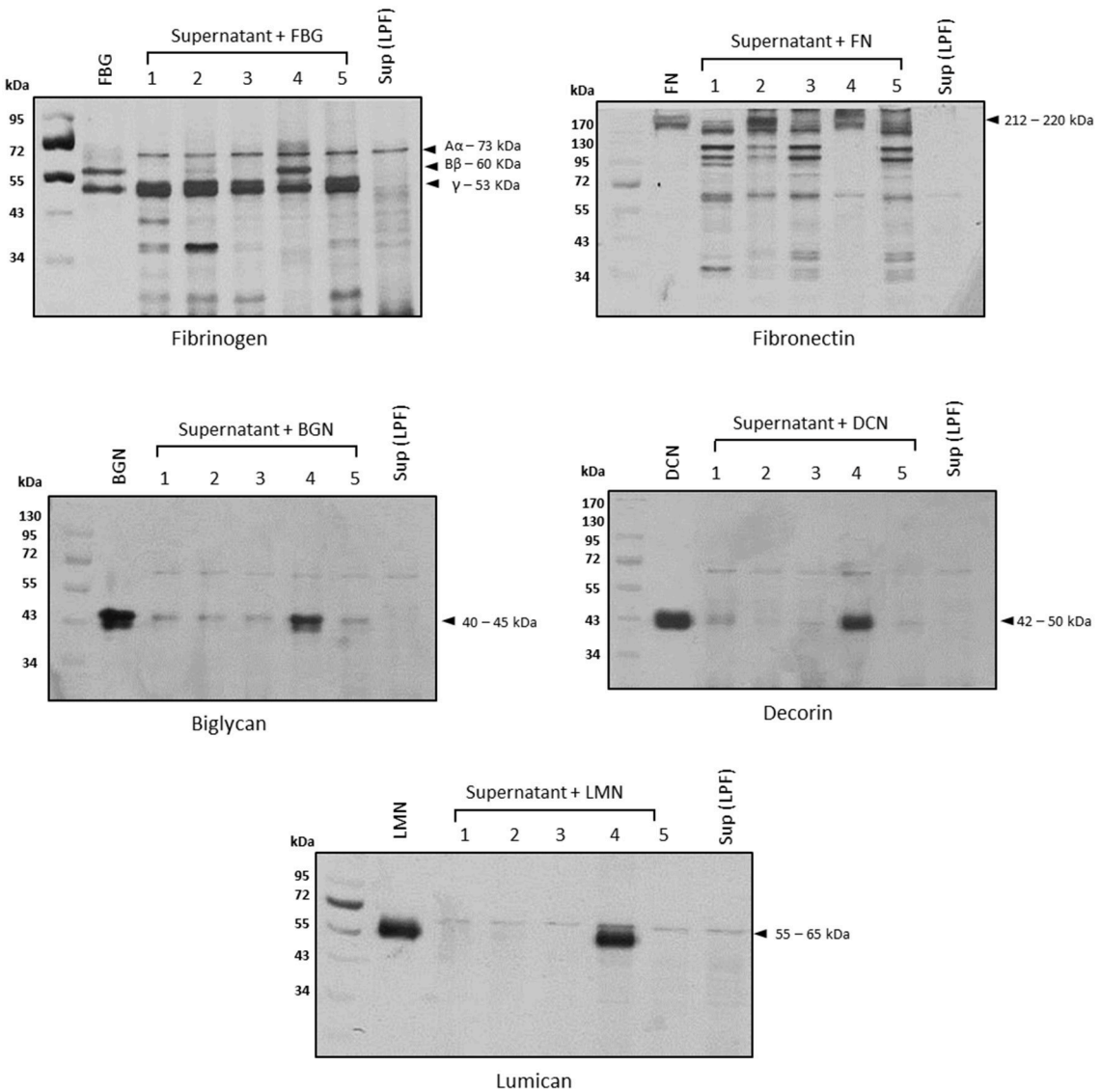


FIGURE 3 | Proteolytic activities are inhibited by 1,10-phenanthroline. Before the addition of each substrate, the supernatant of pathogenic *Leptospira interrogans* serovar Kennewicki strain Fromm (0.5 μg of total secreted proteins) was incubated with inhibitors of serine proteases (5 mmol/L PMSF; lane 2), cysteine proteases (28 μmol/L E-64; lane 3), metalloproteases (5 mmol/L 1,10-phenanthroline; lane 4), or aspartyl proteases (5 μmol/L pepstatin; lane 5) for 30 min at room temperature. Substrates were added and incubations proceeded for 18 h. Cleavage products were analyzed by SDS- polyacrylamide gel under reducing conditions. Gels were silver stained. FBG, fibrinogen; FN, plasma fibronectin; BGN, biglycan; DCN, decorin; LMN, lumican; Sup (LPF), supernatant of *Leptospira interrogans* serovar Kennewicki strain Fromm.

fungi. A number of bacterial proteases contributing to invasion have been described to date. The alkaline protease (AprA), the proteases LasA, LasB, and protease IV from *P. aeruginosa* cause tissue damage during infections by inactivating components of the connective tissue (Schmidtchen et al., 2001). The same applies to Staphopain A (ScpA), aureolysin from *Staphylococcus aureus* and gelatinase (GelE) from *Enterococcus faecalis* which have been shown to degrade collagen, elastin and laminin, and also contribute to immune evasion (Park et al., 2008; Koziel and Potempa, 2013; Senyürek et al., 2014).

Pathogenic *Leptospira* are known to cross tissue barriers and rapidly reach the bloodstream. Target tissues are colonized even before bacterial multiplication (Wunder et al., 2016). Rapid dissemination within the host stems from leptospiral spiral shape associated with a translational motility, allowing them to efficiently swim in viscous gel-like environments (revised in Picardeau, 2017). In addition, proteases released during the initial stages of infection may also contribute to leptospiral invasion and immune evasion (Fraga et al., 2014). In this work, proteolytic activity of secreted leptospiral proteinases against some ECM components and human plasma proteins was

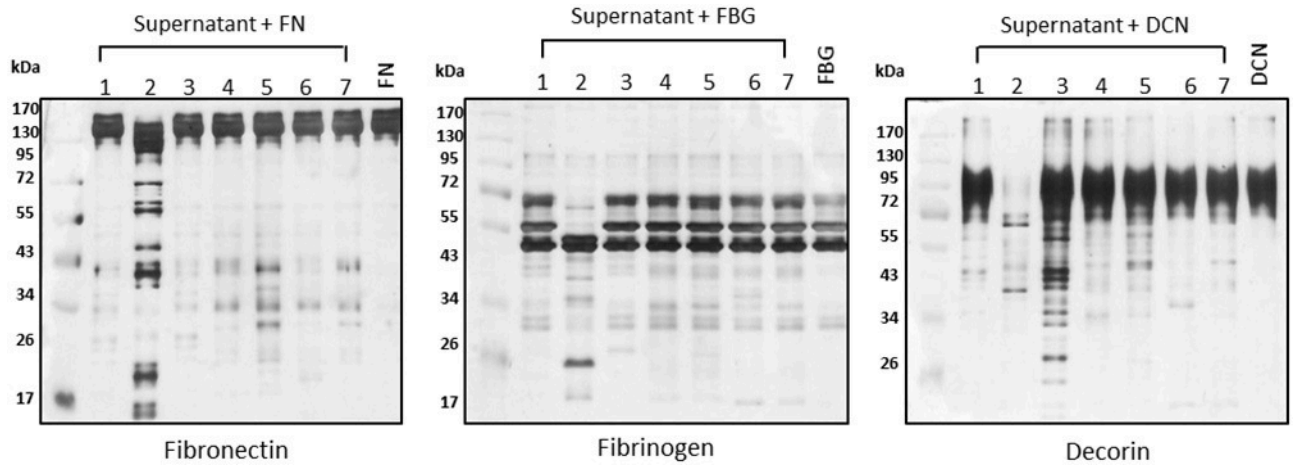


FIGURE 4 | Proteolytic activity correlates with virulence. Supernatants of (1) *L. biflexa* serovar Patoc strain Patoc I, (2) *L. interrogans* serovar Kennewicki strain Fromm (LPF), (3) *L. biflexa* serovar Andamana strain CH11, (4) *L. interrogans* serovar Copenhageni strain 10A, (5) *L. interrogans* serovar Pomona strain Pomona, (6) *L. kirshneri* serovar Cynopteri strain 3522 C, (7) *L. noguchi* serovar Panama CZ 214 (0.5 μg of total secreted proteins) were incubated with plasma fibronectin (5 μg), fibrinogen (15 μg), and decorin (2 μg) for 18 h h at 37°C. Cleavage products were analyzed by SDS- polyacrylamide gel under reducing conditions. Gels were silver stained. FN, plasma fibronectin; FBG, fibrinogen; DCN, decorin. Lane 8: controls (purified substrates).

TABLE 1 | Proteases identified in the *L. interrogans* serovar Kennewicki strain Fromm (LPF) exoproteome.

EMBL GenBank	Protein	Gene	Class
EKO25673.1	Pappalysin-1 domain protein	LEP1GSC104_4629	Metallopeptidase
AAN49383.1	Predicted Zn-dependent protease	*tldD*	Metallopeptidase
AAN49656.2	Methionine aminopeptidase	*map*	Metalloaminopeptidase
EMP06453.1	Papain family cysteine protease	LEP1GSC124_0278	Cysteine peptidase
M3CS93	ATP-dependent protease ATPase subunit HslU	*hslU*	Peptidase
AAN49152.2	ATP-dependent Clp protease proteolytic	*clpP*	Serine endopeptidase

evaluated. Proteases present in the culture supernatant of the virulent LPF strain degraded the small leucine-rich proteoglycans decorin, biglycan and lumican.

Ubiquitously distributed in the connective tissue, decorin interacts with multiple ECM compounds, being essential for matrix integrity. It is also involved in collagen fibrillogenesis, wound repair, angiostasis, tumor growth, and autophagy (Gubbiotti et al., 2016). Biglycan, another SLRP, is present in large quantities in skeletal and hard tissues (Wadhwa et al., 2004), but is also found in the kidneys (Schaefer et al., 2000), liver (Högemann et al., 1997), and skin (Fleischmajer et al., 1991). Like decorin and biglycan, lumican plays important roles in ECM organization and modulates biological processes, including regulation of collagen assembly into fibrils (McEwan et al., 2006; Schaefer and Iozzo, 2008; Stamov et al., 2013), and regulation of apoptosis, cell growth, invasion and angiogenesis (revised in Nikitovic et al., 2014). Interestingly, it has been shown that *L. interrogans* binds to proteoglycans expressed by mammalian cells (Breiner et al., 2009). Glycosaminoglycans (GAGs), covalently linked to proteoglycan core proteins, are the primary targets for leptospiral adhesion, and chondroitin sulfate B is the preferred GAG used for binding (Breiner et al., 2009). As decorin and biglycan are chondroitin sulfate/dermatan sulfate proteoglycans, we can speculate that these GAGs may serve as a platform for *Leptospira* anchoring, and subsequent local degradation of proteoglycan core proteins by bacterial secreted proteases.

On the assumption that basement membranes are also targeted by bacterial proteases, the effect of leptospiral secreted proteases on purified laminin and matrigel was also evaluated. Under our experimental conditions, these two substrates were not degraded. Gelatinolytic activity of *Leptospira* supernatants was evaluated by zymography. Proleotytic activity related to protein bands of approximately 72 kDa and 42 kDa was observed in gels co-polymerized with gelatin. A previous work by Madathiparambil et al. (2011) reported the presence of multiple gelatinases extracted from whole *Leptospira* ranging from 32 to 240 kDa. Some of them, including the ones detected in the present work, may be secreted during infection thus contributing to pathogenesis.

Proteolytic cleavage of plasma fibronectin and fibrinogen was also observed upon incubation with the culture supernatant of *L. interrogans* strain LPF. Purified fibrinogen and fibrinogen in human plasma were dose-dependently degraded by LPF proteases. Fibrinogen, a key clotting protein, plays a crucial role in coagulation and homeostasis. As a consequence of vascular damage, thrombin cleaves fibrinogen into insoluble

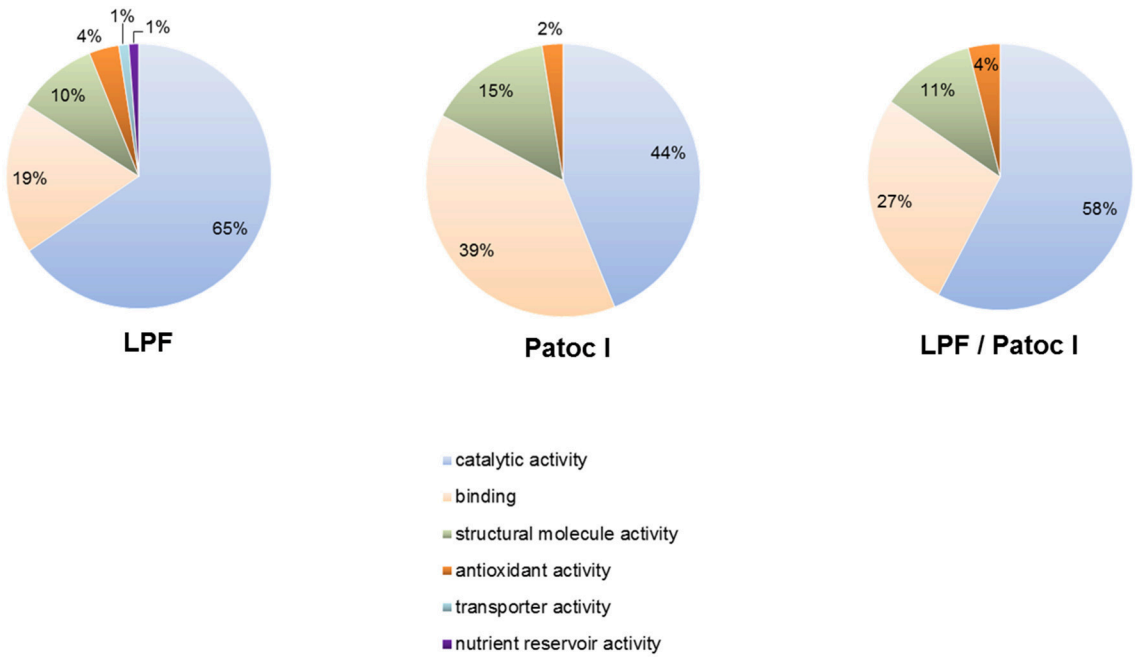


FIGURE 5 | Assignment of secreted proteins to Clusters of Orthologous Groups of proteins (COGs). The proteins identified in *L. interrogans* and *L. biflexa* exoproteomes were classified within the molecular function category. The predicted percentage of proteins in each subcategory is indicated. Pie charts of LPF unique proteins, Patoc I unique proteins and common proteins (LPF/Patoc I) are presented.

fibrin, triggering the fibrin network formation, essential for homeostasis. During infection several pathogens produce proteinases that degrade fibrinogen and, as a consequence, repair of injured sites is retarded (revised in Sun, 2006). One example is the treponemal metalloproteinase pallilysin (Tp0751), which in concert with the serine protease Tp0750, contributes to *Treponema pallidum* dissemination by inhibiting coagulation, promoting fibrinolysis, and degrading ECM components (Houston et al., 2011, 2012, 2014). Inflammation, vascular damage and lung hemorrhage are typical clinical manifestations of patients with severe leptospirosis (revised in Murray, 2015). Studies aiming to investigate whether these pathologies may be caused by fibrinogen-degrading proteinases of *Leptospira* are currently underway.

Thrombin and plasminogen, two other coagulation cascade molecules, were also incubated with *Leptospira* supernatants, and were not susceptible to degradation. It has been previously shown that *Leptospira* has multiple receptors for human plasminogen, and that, once bound to the bacterial surface, this host protease zymogen is converted to its active form, plasmin (Verma et al., 2010; Vieira et al., 2010; Nogueira et al., 2013; Wolff et al., 2013; Castiblanco-Valencia et al., 2016; Salazar et al., 2017). Since bound-plasmin(ogen) degrades ECM components and complement molecules *in vitro* (revised in Fraga et al., 2016), binding intact plasminogen may be more beneficial to the bacterium than degrading this zymogen.

By analyzing the proteolytic capacity of a larger number of strains, we found that culture-attenuated *Leptospira* strains failed to cleave ECM components, leading us to conclude that the proteases involved in this process are produced only under virulence conditions. Since 1,10-phenanthroline was an effective degradation inhibitor, it is presumed that metalloproteases were responsible for the majority of the observed proteolytic activity. Following these observations, proteomic analysis was performed in order to identify secreted proteases of virulent *Leptospira* that may be responsible for the degradation of host components. Among the proteases identified, pappalysin-1 domain protein deserves special attention. This metalloproteinase is well conserved among pathogenic *Leptospira* species according to Analysis Basic Local Alignment Search Tool (BLAST) analysis. A human ortholog, pregnancy-associated plasma protein-A (PAPP-A), was initially described as an abundant placental protein detected in pregnant women. Further studies have shown that PAPP-A is expressed in a variety of tissues and is found tightly bound to GAGs on cell surfaces. This secreted metalloproteinase targets mainly insulin-like growth factor binding proteins (Oxvig, 2015). Functional characterization of *Leptospira* pappalysin-1 domain protein will be the subject of future studies of our group.

Furthermore, several moonlighting proteins were identified (**Supplementary Table 5**), thus endorsing previous exoproteomic data obtained from *Leptospira* culture supernatants (Eshghi et al., 2015). It is worth mentioning that the majority of moonlighting proteins reported by Eshghi and colleagues were also detected in our preparations, and among them are elongation Factor Tu (EF-Tu), enolase, and catalase. EF-Tu and enolase were described as plasminogen binding proteins (Nogueira et al., 2013; Wolff et al., 2013). They also interact with the complement regulators Factor H (EF-Tu)

and C4b binding protein (C4BP) (Wolff et al., 2013; Salazar et al., 2017). Catalase is involved in oxidative stress resistance (Eshghi et al., 2012). By displaying additional functions related to interactions with host cells, some moonlighting proteins including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fructose-1,6-biphosphate aldolase (FBA) are presumed to contribute to bacterial virulence (Henderson, 2017). However, as their canonical functions are generally associated with essential roles within the cell, and knockouts are generally non-viable, the assumption that they may contribute to bacterial virulence sometimes stems purely from *in vitro* experimentation. In addition, 15 out of 25 moonlighting proteins identified in our study were present in the saprophytic strain Patoc I (**Supplementary Table 5**), raising concerns about their role in virulence. For the moment, what can be assumed is that moonlighting proteins contribute to expand the functional proteome of a particular bacterium (Henderson, 2017). An obvious question that arises from our observations is how these and other proteins devoid of N-terminal signal sequence or predicted to be exported through a non-classical secretory pathway are directed to the extracellular space. In line with the findings reported by Eshghi et al. (2015), around 60% of the proteins identified in the present study fall into this category. Whether *Leptospira* possesses particular export mechanisms remains an open question.

In conclusion, leptospiral extracellular proteases display proteolytic activity against proteoglycans and plasma proteins. The capacity to degrade host molecules correlates with *Leptospira* virulence, and may contribute to its dissemination potential.

AUTHOR CONTRIBUTIONS

LS performed most of the experiments and prepared all figures. MM contributed to sample preparation, proteomic data analysis, and aided in interpreting the results. EK contributed to proteomic execution. AO contributed to proteomic data analysis and provided a critical feedback. AA performed some of the experiments. GS provided all *Leptospira* strains used in the study. MH contributed to the analysis and interpretation of the data. LI provided critical feedback and helped shape the research. TF contributed to the design and implementation of the research. SS contributed with reagents, and performed the final proteomic analyses; contributed to the writing of the manuscript. AB conceived the study and was in charge of overall direction and planning, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grants # 2014/00926-3 and 2013/17419-4 (fellowship for LS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00092/full#supplementary-material

Supplementary Figure 1 | Dose-dependent cleavage of purified and plasma fibrinogen. *Leptospira interrogans* serovar Kennewicki strain Fromm (LPF) supernatant (0.01–0.5 μg total secreted proteins) was incubated with 15 μg of purified fibrinogen **(A)** or with human plasma containing an equivalent amount of fibrinogen **(B)** for 2 h at 37°C. Cleavage products were analyzed by SDS-polyacrylamide gel under reducing conditions and gels were silver stained **(A)** or submitted to Western blot and detected with anti-human fibrinogen **(B)**. *cleavage products. FBG, fibrinogen.

Supplementary Table 1 | Identification of proteins in LPF and Patoc I strains by in solution tripsin digestion and LC-MS-MS analysis.

Supplementary Table 2 | Proteins identified only in the LPF strain.

Supplementary Table 3 | Proteins identified only in the Patoc I strain.

Supplementary Table 4 | Proteins identified in the supernatants of both LPF and Patoc I strains.

Supplementary Table 5 | Putative moonlighting proteins identified in LPF and Patoc I culture supernatants.

REFERENCES

Amamura, T. A., Fraga, T. R., Vasconcellos, S. A., Barbosa, A. S., and Isaac, L. (2017). Pathogenic *Leptospira* secreted proteases target the membrane attack complex: a potential role for thermolysin in complement inhibition. *Front. Microbiol.* 8:958. doi: 10.3389/fmicb.2017.00958

Barbosa, A. S., Abreu, P. A., Vasconcellos, S. A., Morais, Z. M., Gonçales, A. P., Silva, A. S., et al. (2009). Immune evasion of *Leptospira* species by acquisition of human complement regulator C4BP. *Infect. Immun.* 77, 1137–1143. doi: 10.1128/IAI.01310-08

Barbosa, A. S., Monaris, D., Silva, L. B., Morais, Z. M., Vasconcellos, S. A., Cianciarullo, A. M., et al. (2010). Functional characterization of LcpA, a surface-exposed protein of *Leptospira* spp. that binds the human complement regulator C4BP. *Infect. Immun.* 78, 3207–3216. doi: 10.1128/IAI.00279-10

Breiner, D. D., Fahey, M., Salvador, R., Novakova, J., and Coburn, J. (2009). *Leptospira* interrogans binds to human cell surface receptors including proteoglycans. *Infect. Immun.* 77, 5528–5536. doi: 10.1128/IAI.00546-09

Castiblanco-Valencia, M. M., Fraga, T. R., Pagotto, A. H., Serrano, S. M., Abreu, P. A., Barbosa, A. S., et al. (2016). Plasmin cleaves fibrinogen and the human complement proteins C3b and C5 in the presence of *Leptospira interrogans* proteins: a new role of LigA and LigB in invasion and complement immune evasion. *Immunobiology* 221, 679–689. doi: 10.1016/j.imbio.2016.01.001

Chagnot, C., Listrat, A., Astruc, T., and Desvaux, M. (2012). Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components. *Cell Microbiol.* 14, 1687–1696. doi: 10.1111/cmi.12002

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. doi: 10.1038/nbt.1511

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V., and Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794–1805. doi: 10.1021/pr101065j

Eshghi, A., Lourdault, K., Murray, G. L., Bartpho, T., Sermswan, R. W., Picardeau, M., et al. (2012). *Leptospira interrogans* catalase is required for resistance to H_2O_2 and for virulence. *Infect. Immun.* 80, 3892–3899. doi: 10.1128/IAI.00466-12

Eshghi, A., Pappalardo, E., Hester, S., Thomas, B., Pretre, G., and Picardeau, M. (2015). Pathogenic *Leptospira interrogans* exoproteins are primarily involved in heterotrophic processes. *Infect. Immun.* 83, 3061–3073. doi: 10.1128/IAI.00427-15

Fleischmajer, R., Fisher, L. W., MacDonald, E. D., Jacobs, L. Jr., Perlish, J. S., and Termine, J. D. (1991). Decorin interacts with fibrillar collagen of embryonic and adult human skin. *J. Struct. Biol.* 106, 82–90. doi: 10.1016/1047-8477(91)90065-5

Fraga, T. R., Courrol Ddos, S., Castiblanco-Valencia, M. M., Hirata, I. Y., Vasconcellos, S. A., Juliano, L., et al. (2014). Immune evasion by pathogenic *Leptospira* strains: the secretion of proteases that directly cleave complement proteins. *J. Infect. Dis.* 209, 876–886. doi: 10.1093/infdis/jit569

Fraga, T. R., Isaac, L., and Barbosa, A. S. (2016). Complement evasion by pathogenic *Leptospira*. *Front. Immunol.* 7:623. doi: 10.3389/fimmu.2016.00623

Ghilarov, D., Serebryakova, M., Stevenson, C. E. M., Hearnshaw, S. J., Volkov, D. S., Maxwell, A., et al. (2017). The origins of specificity in the microcin-processing protease TldD/E. *Structure* 25, 1549–1561.e5. doi: 10.1016/j.str.2017.08.006

Gubbiotti, M. A., Vallet, S. D., Ricard-Blum, S., and Iozzo, R. V. (2016). Decorin interacting network: a comprehensive analysis of decorin-binding partners and their versatile functions. *Matrix Biol.* 55,7–21. doi: 10.1016/j.matbio.2016.09.009

Hashimoto, V. L., Abreu, P. A., Carvalho, E., Gonçales, A. P., Morais, Z. M., Vasconcellos, S. A., et al. (2013). Evaluation of the elastinolytic activity and protective effect of Leptallo, I., a protein composed by metalloprotease and FA5/8C domains, from *Leptospira interrogans* Copenhageni. *Microb. Pathog.* 61–62, 29–36. doi: 10.1016/j.micpath.2013.04.011

Henderson, B. (2017). *Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections*. Hoboken, NJ: Wiley-Blackwell

Högemann, B., Edel, G., Schwarz, K., Krech, R., and Kresse, H. (1997). Expression of biglycan, decorin and proteoglycan-100/CSF-1 in normal and fibrotic human liver. *Pathol. Res. Pract.* 193, 747–751. doi: 10.1016/S0344-0338(97)80052-0

Houston, S., Hof, R., Francescutti, T., Hawkes, A., Boulanger, M. J., and Cameron, C. E. (2011). Bifunctional role of the *Treponema pallidum* extracellular matrix binding adhesin Tp0751. *Infect. Immun.* 79, 1386–1398. doi: 10.1128/IAI.01083-10

Houston, S., Hof, R., Honeyman, L., Hassler, J., and Cameron, C. E. (2012). Activation and proteolytic activity of the *Treponema pallidum* metalloprotease, pallilysin. *PLoS Pathog.* 8:e1002822. doi: 10.1371/journal.ppat.1002822

Houston, S., Russell, S., Hof, R., Roberts, A. K., Cullen, P., Irvine, K., et al. (2014). The multifunctional role of the pallilysin-associated *Treponema pallidum* protein, Tp0750, in promoting fibrinolysis and extracellular matrix component degradation. *Mol. Microbiol.* 91, 618–634. doi: 10.1111/mmi.12482

Kassegne, K., Hu, W., Ojcius, D. M., Sun, D., Ge, Y., Zhao, J., et al. (2014). Identification of collagenase as a critical virulence factor for invasiveness and transmission of pathogenic *Leptospira* species. *J. Infect. Dis.* 209, 1105–1115. doi: 10.1093/infdis/jit659

Koziel, J., and Potempa, J. (2013). Protease-armed bacteria in the skin. *Cell Tissue Res.* 351, 325–337. doi: 10.1007/s00441-012-1355-2

Levett, P. N. (2001). Leptospirosis. *Clin. Microbiol. Rev.* 14, 296–326. doi: 10.1128/CMR.14.2.296-326.2001

Madathiparambil, M. G., Cattavarayane, S., Manickam, G. D., Singh, K., Perumana, S. R., and Sehgal, S. C. (2011). A zymography analysis of proteinase activity present in *Leptospira*. *Curr. Microbiol.* 62, 917–922. doi: 10.1007/s00284-010-9810-3

Mani, M., Chen, C., Amblee, V., Liu, H., Mathur, T., Zwicke, G., et al. (2015). MoonProt: a database for proteins that are known to moonlight. *Nucleic Acids Res.* 43, D277-D282. doi: 10.1093/nar/gku954

McEwan, P. A., Scott, P. G., Bishop, P. N., and Bella, J. (2006). Structural correlations in the Family of small leucine-rich repeat proteins and proteoglycans. *J. Struct. Biol.* 155, 294–305. doi: 10.1016/j.jsb.2006.01.016

Meri, T., Murgia, R., Stefanel, P., Meri, S., and Cinco, M. (2005). Regulation of complement activation at the C3-level by serum resistant leptospires. *Microb. Pathog.* 39, 139–147. doi: 10.1016/j.micpath.2005.07.003

Murray, G. L. (2015). The molecular basis of leptospiral pathogenesis. *Curr. Top. Microbiol. Immunol.* 387, 139–185. doi: 10.1007/978-3-662-45059-8_7

Nikitovic, D., Kouvidi, K., Voudouri, K., Berdiaki, A., Karousou, E., Passi, A., et al. (2014). The motile breast cancer phenotype roles of proteoglycans/glycosaminoglycans. *Biomed. Res. Int.* 2014:124321. doi: 10.1155/2014/124321

Nogueira, S. V., Backstedt, B. T., Smith, A. A., Qin, J. H., Wunder, E. A. Jr., Ko, A., et al. (2013). *Leptospira interrogans* enolase is secreted extracellularly and interacts with plasminogen. *PLoS ONE* 8:e78150. doi: 10.1371/journal.pone.0078150

Oxvig, C. (2015). The role of PAPP-A in the IGF system: location, location, location. *J. Cell Commun. Signal.* 9, 177–187. doi: 10.1007/s12079-015-0259-9.

Park, S. Y., Shin, Y. P., Kim, C. H., Park, H. J., Seong, Y. S., Kim, B. S., et al. (2008). Immune evasion of *Enterococcus faecalis* by an extracellular gelatinase that cleaves C3 and iC3b. *J. Immunol.* 181, 6328–6336. doi: 10.4049/jimmunol.181.9.6328

Picardeau, M. (2017). Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat. Rev. Microbiol.* 15, 297–307. doi: 10.1038/nrmicro.2017.5

Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* 2, 1896–1906. doi: 10.1038/nprot.2007.261

Salazar, N., Souza, M. C., Biasioli, A. G., Silva, L. B., and Barbosa, A. S. (2017). The multifaceted roles of *Leptospira* enolase. *Res. Microbiol.* 168, 157–164. doi: 10.1016/j.resmic.2016.10.005

Schaefer, L., Gröne, H. J., Raslik, I., Robenek, H., Ugorcakova, J., Budny, S., et al. (2000). Small proteoglycans of normal adult human kidney: distinct expression. patterns of decorin, biglycan, fibromodulin, and lumican. *Kidney Int.* 58, 1557–1568. doi: 10.1046/j.1523-1755.2000.00317.x

Schaefer, L., and Iozzo, R. V. (2008). Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J. Biol. Chem.* 283, 21305–21309. doi: 10.1074/jbc.R800020200

Schmidtchen, A., Wolff, H., and Hansson, C. (2001). Differential proteinase expression by *Pseudomonas aeruginosa* derived from chronic leg ulcers. *Acta Derm. Venereol.* 81, 406–409. doi: 10.1080/000155501317208336

Senyürek, I., Kempf, W. E., Klein, G., Maurer, A., Kalbacher, H., Schäfer, L., et al. (2014). Processing of laminin α chains generates peptides involved in wound healing and host defense. *J. Innate Immun.* 6, 467–484. doi: 10.1159/000357032

Stamov, D. R., Müller, A., Wegrowski, Y., Brezillon, S., and Franz, C. M. (2013). Quantitative analysis of type I collagen fibril regulation by lumican and decorin using AFM. *J. Struct. Biol.* 183, 394–403. doi: 10.1016/j.jsb.2013.05.022

Subramanian, S. (1984). Dye-ligand affinity chromatography: the interaction of Cibacron Blue F3GA with proteins and enzymes. *CRC Crit. Rev. Biochem.* 16, 169–205. doi: 10.3109/10409238409102302

Sun, H. (2006). The interaction between pathogens and the host coagulation system. *Physiology (Bethesda)* 21, 281–288. doi: 10.1152/physiol.00059.2005

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., et al. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* 13, 731–740. doi: 10.1038/nmeth.3901

Verma, A., Brissette, C. A., Bowman, A. A., Shah, S. T., Zipfel, P. F., and Stevenson, B. (2010). Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect. Immun.* 78, 2053–2059. doi: 10.1128/IAI.01282-09

Vieira, M. L., Atzingen, M. V., Oliveira, T. R., Oliveira, R., Andrade, D. M., Vasconcellos, S. A., et al. (2010). *In vitro* identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS ONE* 5:e11259. doi: 10.1371/journal.pone.0011259

Wadhwa, S., Embree, M. C., Bi, Y., and Young, M. F. (2004). Regulation, regulatory activities, and function of biglycan. *Crit. Rev. Eukaryot. Gene Expr.* 14, 301–315. doi: 10.1615/CritRevEukaryotGeneExpr.v14.i4.50

Wiśniewski, J. R., Zougman, A., Nagaraj, N., and Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nat. Methods* 6, 359–362. doi: 10.1038/nmeth.1322

Wolff, D. G., Castiblanco-Valencia, M. M., Abe, C. M., Monaris, D., Morais, Z. M., Souza, G. O., et al. (2013). Interaction of *Leptospira* elongation factor Tu with plasminogen and complement factor H: a metabolic leptospiral protein with moonlighting activities. *PLoS ONE* 8:e81818. doi: 10.1371/journal.pone.0081818

Wunder, E. A. Jr., Figueira, C. P., Santos, G. R., Lourdault, K., Matthias, M. A., Vinetz, J. M., et al. (2016). Real-Time PCR reveals rapid dissemination of *Leptospira interrogans* after intraperitoneal and conjunctival inoculation of hamsters. *Infect. Immun.* 84, 2105–2115. doi: 10.1128/IAI.00094-16

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 20 June 2018
doi: 10.3389/fcimb.2018.00204

Leptospirosis Pathophysiology: Into the Storm of Cytokines

Julie Cagliero[1], *Sharon Y. A. M. Villanueva*[2] *and Mariko Matsui*[1]*

[1] *Group Immunity and Inflammation, Institut Pasteur International Network, Institut Pasteur in New Caledonia, Nouméa, New Caledonia,* [2] *Department of Medical Microbiology, College of Public Health, University of the Philippines, Manila, Philippines*

Leptospirosis is a neglected tropical zoonosis caused by pathogenic spirochetes of the genus *Leptospira*. Infected reservoir animals, typically mice and rats, are asymptomatic, carry the pathogen in their renal tubules, and shed pathogenic spirochetes in their urine, contaminating the environment. Humans are accidental hosts of pathogenic *Leptospira*. Most human infections are mild or asymptomatic. However, 10% of human leptospirosis cases develop into severe forms, including high leptospiremia, multi-organ injuries, and a dramatically increased mortality rate, which can relate to a sepsis-like phenotype. During infection, the triggering of the inflammatory response, especially through the production of cytokines, is essential for the early elimination of pathogens. However, uncontrolled cytokine production can result in a cytokine storm process, followed by a state of immunoparalysis, which can lead to sepsis and associated organ failures. In this review, the involvement of cytokine storm and subsequent immunoparalysis in the development of severe leptospirosis in susceptible hosts will be discussed. The potential contribution of major pro-inflammatory cytokines in the development of tissue lesions and systemic inflammatory response, as well as the role of anti-inflammatory cytokines in contributing to the onset of a deleterious immunosuppressive cascade will also be examined. Data from studies comparing susceptible and resistant mouse models will be included. Lastly, a concise discussion on the use of cytokines for therapeutic purposes or as biomarkers of leptospirosis severity will be provided.

Keywords: ***Leptospira*, leptospirosis, inflammatory response, cytokine storm, immunoparalysis, susceptible/resistant hosts**

OPEN ACCESS

Edited by:
Elsio Augusto Wunder Jr., Yale University, United States

Reviewed by:
Maria Gomes-Solecki, University of Tennessee Health Science Center, United States
Lourdes Isaac, Universidade de São Paulo, Brazil

***Correspondence:**
Mariko Matsui
mmatsui@pasteur.nc

Received: *28 February 2018*
Accepted: *31 May 2018*
Published: *20 June 2018*

Citation:
Cagliero J, Villanueva SYAM and Matsui M (2018) Leptospirosis Pathophysiology: Into the Storm of Cytokines. Front. Cell. Infect. Microbiol. 8:204. doi: 10.3389/fcimb.2018.00204

INTRODUCTION

Leptospirosis is a re-emerging neglected zoonosis, caused by pathogenic spirochete bacteria from the genus *Leptospira* and estimated to infect more than a million people with approximately 60,000 deaths annually (Costa et al., 2015a,b; Picardeau, 2015). Number of fatal cases is comparable to or even higher than some other important neglected tropical diseases such as severe dengue or visceral leishmaniasis (Picardeau, 2015). Leptospirosis occurs after direct or indirect contact with bacteria shed in the urine of reservoir animals, mostly rodents, in which infection is asymptomatic and results in chronic renal carriage (Adler and de la Pena Moctezuma, 2010). Clinical manifestations in infected humans are extremely variable, including flu-like symptoms, and spontaneously resolve in 90% of cases (Haake and Levett, 2015; Gomes-Solecki et al., 2017). However, 10% of patients develop severe forms of the disease and are designated as susceptible hosts in this review. Severe human leptospirosis is characterized by multi-organ failures and is associated with a dramatic increase in the mortality rate (Adler and de la Pena Moctezuma, 2010; Haake and Levett, 2015).

Hepatic dysfunctions associated with renal failure and hemorrhages constitute Weil's syndrome, a severe form of leptospirosis (Haake and Levett, 2015). Acute kidney injury (AKI) is commonly reported as an early manifestation of acute leptospirosis and could possibly evolve to chronic kidney disease (CKD) (Correa-Rotter et al., 2014; Herath et al., 2014). Severe pulmonary hemorrhagic syndrome (SPHS) with acute respiratory distress syndrome (ARDS) can also occur and can be confused with viral pneumonitis (Trevejo et al., 1998; Haake and Levett, 2015).

Although it is still unclear why leptospirosis patients present with various clinical manifestations, both innate and adaptive immune responses to *Leptospira* infection influence the outcome of the disease. Induction of an inflammatory response due to an infection with a pathogen can initiate destructive immune mechanisms leading to host tissue damages, sepsis and death. Interestingly, the cytokine storm process was suggested to have a role in the development of severe leptospirosis, especially in humans (Reis et al., 2013; Haake and Levett, 2015). In this review, we summarized evidences supporting this hypothesis, mainly based on results from studies on hosts susceptible and resistant to lethal infection.

INFLAMMATORY RESPONSE TO *LEPTOSPIRA* INFECTION

During an infection, contact with pathogens activates the innate immune system by generating an inflammatory response. Microbial Pathogen-Associated Molecular Patterns (PAMPs) will be recognized by the Pattern Recognition Receptors (PRRs) expressed at the surface of innate immune cells, mainly the Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Akira et al., 2006). The PAMPs/PRR association triggers an inflammatory cascade by activating multiple intracellular signaling pathways, including the NF-κB and activator protein 1 (AP-1) transcription factors (Schroder and Tschopp, 2010), which in turn regulate the expression of cytokines, Prostaglandins (PGs), and Nitric Oxide (NO) (Tisoncik et al., 2012; Turner et al., 2014). PGs and NO are pro-inflammatory molecules that increase arterial dilation and vascular permeability, both being key events required for the influx of immune cells (Ricciotti and FitzGerald, 2011; Wink et al., 2011). Pro-inflammatory cytokines include interleukins (IL)-1β, IL-6, IL-12, interferons (IFNs) and tumor necrosis factors (TNFs), as well as chemokines, which act as chemoattractants to recruit leucocytes to the site of tissue damage and/or infection (Tisoncik et al., 2012; Turner et al., 2014). Interestingly, AP-1 and NF-κB also modulate the pro-inflammatory response through the induction of immunomodulatory cytokines as IL-4, IL-10, IL-13 or Transforming Growth Factor-β (TGF-β) acting in concert with cytokines inhibitors to offset the massive induction of pro-inflammatory mediators (Dinarello, 2007; Turner et al., 2014).

Data show that leptospiral PAMPs stimulate innate immunity through several PRRs to produce cytokines and activate the inflammatory cascade, as was recently reviewed by C. Werts (Werts, 2017). Mechanisms underlying the specific leptospiral PAMPs/PRR-triggered inflammation have not yet been fully elucidated. Interestingly, in contrast to other bacterial LPS (e.g., from *E. coli*) that classically activates TLR4 signaling pathway, leptospiral LPS is not recognized by TLR4, but by TLR2 in human cells, while both TLR2 and TLR4 are activated in mice (Nahori et al., 2005). Moreover, TLR4-deficient mice develop clinical signs of severe leptospirosis (Gomes-Solecki et al., 2017), which show that murine TLR4 protect mice from developing leptospirosis. This species specificity in leptospiral PAMPs sensing also suggest that hosts could trigger different inflammatory responses according to their susceptibility to leptospirosis.

CYTOKINE STORM IN SEVERE LEPTOSPIROSIS

Inflammatory cytokines and cytokine regulators interact in a complex network finely controlled to clear the pathogens without excessive inflammation-induced organ damage. Indeed, severe infectious diseases are often associated with a prolonged increase in pro-inflammatory IL-1β, TNF-α, IL-6 expression, or "cytokine storm," causing persistent inflammation and followed by a massive and systemic production of anti-inflammatory cytokines, causing a state of "immunoparalysis" (Tisoncik et al., 2012; Zhao et al., 2015). Consequently, tissue edema impairs local organ perfusion, which can result in loss of organ function; furthermore, prolonged endothelial permeabilization can lead to pathogen invasion into the bloodstream and result in a sepsis-like syndrome (Cohen, 2002; Tisoncik et al., 2012). In fact sepsis is now defined as a life-threatening organ dysfunction caused by a dysregulated host response to an infection (Singer et al., 2016) and is referred to as a "cytokine storm-induced syndrome" (Chousterman et al., 2017).

Clinical signs of severe leptospirosis in susceptible hosts include increased leptospiremia and multi-organ failure, specifically affecting kidneys, liver and lungs (Levett, 2001; Haake and Levett, 2015; Yilmaz et al., 2015). Interestingly, these clinical features meet the criterions for a sepsis diagnosis, which suggest that development of severe leptospirosis could be associated with a dysregulated inflammation. Interestingly, data obtained from clinical studies support this hypothesis. The very first quantifications of cytokines in human leptospirosis showed significant increase in TNF-α level from patient sera (Estavoyer et al., 1991; Tajiki and Salomão, 1996; Tajiki et al., 1997). Further investigations reported higher production of cytokines in severe compared to mild disease (Reis et al., 2013; Mikulski et al., 2015; Chirathaworn et al., 2016). Serum levels of pro-inflammatory IL-6, chemokine IL-8 and anti-inflammatory IL-10 were significantly higher among patients that developed SPHS compared to non-SPHS leptospirosis patients (Reis et al., 2013). In addition, concentrations of these cytokines were elevated in sera from patients with organ dysfunction compared to mild cases without organ involvement (Chirathaworn et al., 2016). Experimental infection also showed evidence of differential pattern of cytokine expression depending on resistance or susceptibility of animal models (**Figure 1**). Strictly regulated

induction of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and chemokines CXCL10/IP-10 and CCL3/MIP-1α in resistant mice contrasted with delayed and massive overexpression in susceptible hamsters developing severe tissue lesions (Matsui et al., 2011). Overexpression of anti-inflammatory IL-10 was faster and at higher levels in resistant mice than in hamsters (Matsui et al., 2017). Moreover, expression of TNF-α, IL-1α, and IL-10 were significantly higher in lethally infected hamsters compared to survivors (Vernel-Pauillac and Goarant, 2010). Based on these, dramatic imbalance in the cytokine production upon *Leptospira* infection might play a considerable role in the development of severe leptospirosis.

ROLE OF CYTOKINES IN THE PATHOPHYSIOLOGY OF SEVERE LEPTOSPIROSIS

Pro-inflammatory Cytokines

Although TNF-α was the first cytokine to be associated with leptospirosis severity (Estavoyer et al., 1991; Tajiki and Salomão, 1996), its precise role in the disease pathophysiology is still under debate. Macrophage apoptosis induced by *L. interrogans in vitro* is triggered via caspase-8 and caspase-3-dependant pathways (Jin et al., 2009), and *Leptospira* were suggested to induce apoptosis in organs though the caspase-3-dependent pathway in infected mice (Marinho et al., 2015). Thus, TNF-α might play a role in *Leptospira*-induced apoptosis as it can activate caspase-3- and caspase-8-associated apoptosis (Zhao et al., 2001; Wang et al., 2008).

Kyriakidis et al. reported that TNF-α was the only cytokine found associated with SHPS in leptospirosis patients (Kyriakidis et al., 2011). Alteration of sodium channel was suggested as a cause for the pulmonary damages observed in susceptible *Leptospira*-infected hamsters (Andrade et al., 2007). Interestingly, TNF-α, together with IL-1β, regulate the expression of sodium channel sub-units *in vitro*, which could be part of pulmonary damages underlying mechanism (Yamagata et al., 2009).

Pro-inflammatory IL-6 is induced by TNF-α and IL-1β and produced by a large number of immune and non-immune cells. High concentration of IL-6 is an indicator of septic shock and correlates to leptospirosis severity and SPHS (Reis et al., 2013; Schulte et al., 2013; Papa and Kotrotsiou, 2015). As TNF-α and IL-1β, IL-6 can also activate the coagulation system, especially in endotoxemic models (Schulte et al., 2013). This could relate to clinical bleedings, especially in SHPS (Haake and Levett, 2015), and activation of coagulation (Wagenaar et al., 2010) observed in severe leptospirosis.

High levels of chemokines are found in susceptible hamsters (Matsui et al., 2016), and are associated with organ damage and poor outcome (Reis et al., 2013; Papa and Kotrotsiou, 2015). Notably, higher level of CXCL8/IL-8 expression was found in patients with severe clinical signs and was associated with mortality (Wagenaar et al., 2009a,b). Moreover, high levels of adhesion molecules, ICAM-1 or VCAM, are also associated with leptospirosis-induced organ damages (Del Carlo Bernardi et al., 2012). Chemokines along with endothelial adhesion molecules are induced by TNF-α, which promotes leukocytes attraction and extravasation into injured tissue. Their prolonged production can also promote endothelial barrier disruption and diapedesis, which could explain hemorrhages and immune cells infiltrations in tissues associated with severe leptospirosis.

Together with IL-1β, TNF-α promote the activation of immune cells as macrophages, and subsequent secretion of immuno-regulators including pro-inflammatory factors that amplify the inflammatory response. Moreover, high TNF-α and IL-1 circulating levels are considered as the hallmarks of a cytokine storm and their role in sepsis pathophysiology has been largely investigated (Schulte et al., 2013). These data place TNF-α and IL-1β as master conductors of the awry inflammatory response and the subsequent cytokine storm-induced sepsis observed in severe leptospirosis. However, it also emphasizes that these cytokines may not be the best candidates for investigating specific immune responses to a *Leptospira* infection and for selection of biomarkers for severity prognosis. Genetic studies showing no correlation between TNF-α polymorphisms and leptospirosis outcome are in good accordance with this hypothesis (Lingappa et al., 2004).

Anti-inflammatory and Immunomodulatory Cytokines

Following the onset of a cytokine storm, systemic production of IL-10 is considered as a hallmark of an attempt to restore immunological homeostasis (Tisoncik et al., 2012). High level of IL-10 in septic patients significantly correlates with sepsis and death suggesting profound immunosuppression (Gogos et al., 2000). It also influences the clearance of microorganisms in the host (Duell et al., 2012). In Lyme borreliosis, a disease due to an infection by another spirochete, *Borrelia burgdorferi*, contribution of IL-10 in the attenuation of tissue lesions was shown through the modulation of pro-inflammatory cytokines (Brown et al., 1999; Gautam et al., 2011). Inhibition of endogenous IL-10 also enhanced *Borrelia* elimination (Brown et al., 1999).

In human leptospirosis, controversial results showed either higher levels of IL-10 (Reis et al., 2013; Chirathaworn et al., 2016) or no significant difference in IL-10 levels (Wagenaar et al., 2009a; Mikulski et al., 2015) between mild and severe forms. However, asymptomatic leptospirosis presented an anti-inflammatory response with higher IL-10-producing CD4+ T-cells compared to patients with severe or mild leptospirosis (Volz et al., 2015). Differences in time of sampling might explain this discrepancy. Interestingly, in a murine Cecal Ligation and Puncture (CLP)-induced sepsis model, the inhibition of IL-10 at the time of CLP worsened mortality rate whereas it increased survival when administered 12 h later (Song et al., 1999). Thus, IL-10 contribution in leptospirosis pathophysiology probably depends on the stage and severity of the disease. Altogether, these findings suggest that IL-10 could either be protective by counterbalancing pro-inflammatory cytokines during the early stage of the disease, or have harmful effect in the leptospirosis outcome acting on bacterial burden. Finally, genetic variations could also contribute to the IL-10 dual role since IL-10 promoter

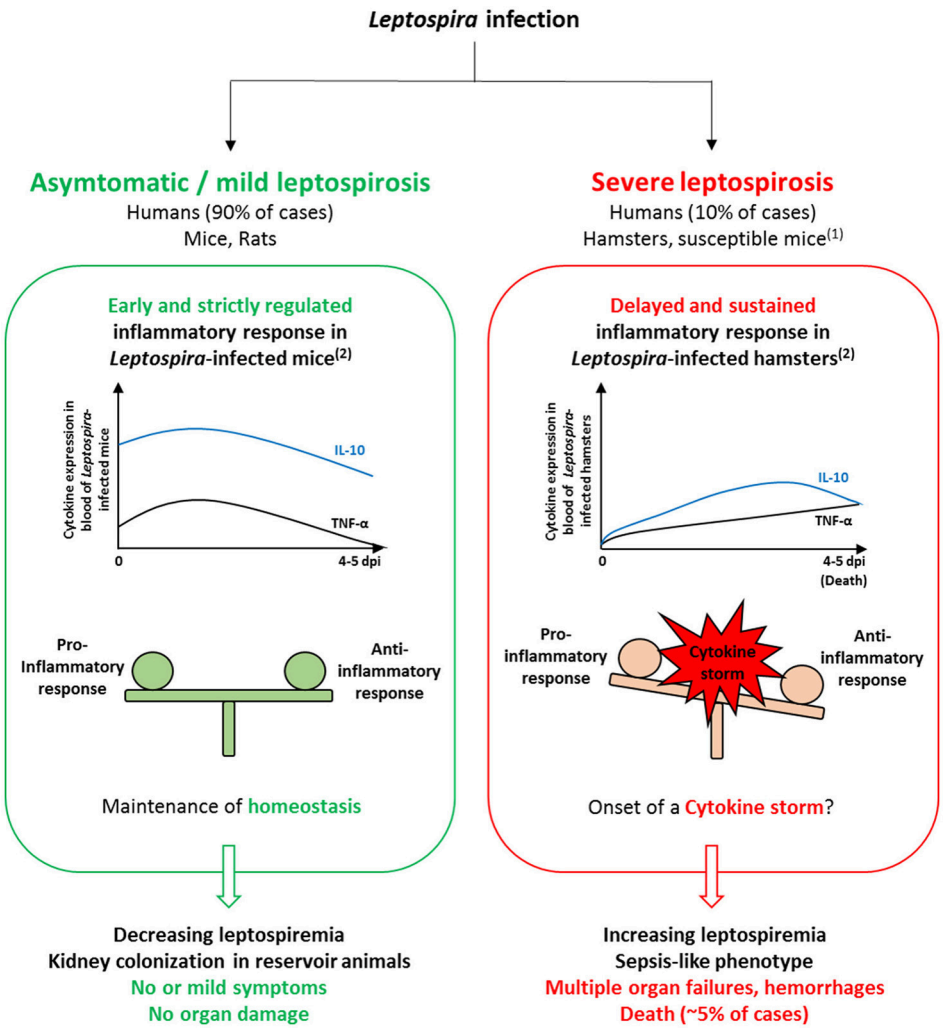


FIGURE 1 | Comparison of models of *Leptospira* infection in resistant and susceptible hosts suggests the importance of the inflammatory response in influencing the disease outcome. In *Leptospira*-infected mice, the inflammatory response is rapid and strictly regulated. Consequently, homeostasis is maintained as bacteria are rapidly cleared from the blood and no or only mild symptoms develop, which relate to mild leptospirosis observed in 90% of human infections. In *Leptospira*-infected hamsters, cytokine production is delayed and sustained, which could trigger the onset of a cytokine storm and impair pathogen clearance from the blood. The resulting sepsis-like phenotype could be associated to increased bacterial load, organ failures and hemorrhages found in infected hamsters as well as in severe forms of leptospirosis in humans. (1)Susceptible mouse models (e.g., C3H/HeJ strain) comprise inbred wild-type, immunosuppressed and transgenic mice, used as models for sublethal and lethal leptospirosis (see Gomes-Solecki et al., 2017 for review). (2)Comparison of the inflammatory response in resistant vs. susceptible animal models is based on expression profiles found in Matsui et al. (2011). Expression levels of IL-10 and TNF-α are representative of anti- and pro-inflammatory cytokine production, respectively.

polymorphisms were associated with lower LPS-dependent IL-10 production and with the development of sepsis in patients with major trauma (Zeng et al., 2009).

Inflammatory response is also restricted by regulatory cytokines, as IL-4 or IL-13, promoting T helper (Th) lymphocyte differentiation toward Th2 lineage, suppressing tissue-damaging effects of sustained inflammation (Opal and Depalo, 2000). Moreover, aberrant production of these cytokines facilitates pathogen invasion during infection (Wynn, 2015). IL-4 is induced during leptospirosis with late but massive overexpression in blood from experimentally infected hamsters (Vernel-Pauillac and Merien, 2006). Serum levels of IL-4 were also increased in human patients (Reis et al., 2013) and frequencies in IL-4 and IL-4R receptor gene polymorphisms were significantly higher in leptospirosis patients compared to healthy subjects (Fialho et al., 2009). Similarly to IL-10, the role of IL-4 in sepsis is still under debate (Chousterman et al., 2017)

Thus, induction of IL-4 and IL-10 might play an important role in leptospirosis pathophysiology that still needs to be precisely described. Indeed, these cytokines could relate to impaired bacterial clearance by restricting pro-inflammatory responses and consequently triggering immunoparalysis related to fulminant septicemia in human leptospirosis (Tisoncik et al., 2012; Zhao et al., 2015).

RESISTANT PHENOTYPE TO *LEPTOSPIRA* INFECTION: ROLE OF THE IMMUNE RESPONSE

Although hamsters and guinea pigs are suitable models to study acute leptospirosis, the cost of their handling and the lack of experimental tools have limited their use in laboratories. As mentioned above, mice, which are much easier to handle, are resistant to *Leptospira* infection and become chronic carrier of the bacterium. However, variable degrees of susceptibility have been observed using different mice strains, which allowed scientists to consider mice as suitable models for studying severe leptospirosis. Consequently, a large number of transgenic, mutant, or immunosuppressed mice are now being used as models of chronic, sub-lethal and lethal leptospirosis (see Gomes-Solecki et al., 2017 for review).

Contrasting with delayed and uncontrolled cytokine production observed in susceptible hosts, the inflammatory response in resistant models is characterized by an early but tightly regulated induction of pro-inflammatory cytokines, and a fast and high overexpression of the anti-inflammatory IL-10 (**Figure 1**). Recently, higher chemokines levels including CCL5/RANTES and CCL8/MCP-2 were quantified in organs from resistant (BALB/c) compared to susceptible (TLR4-defective C3H/HeJ) mice 24 h post-infection (Domingos et al., 2017). Moreover, high expression of chemokines CXCL1/KC, CXCL2/MIP-2, CCL5/RANTES, as well as IL-1β, TNF-α, and IL-10 was observed in sub-lethally infected C3H/HeJ mice (Richer et al., 2015; Sullivan et al., 2017). This phenotype specificity in the immune response is supported by previous data showing that expression of TNF-α and of CXCL2/MIP-2 chemokine is delayed in organs from susceptible mice (da Silva et al., 2012). Thus, time of induction of chemokines and key cytokines seems to be determinant for the development of a resistant phenotype to *Leptospira* infection.

Moreover, and supporting the possible dual role of IL-10, recent studies reported improved bacterial clearance in mouse kidneys in the absence of IL-10 compared to control animals (Devlin et al., 2017; Matsui et al., 2017). Interestingly, IL-10 deficiency did not affect *Leptospira*-dependent induction of TNF-α and IL-6 while it enhanced IL-1β and IFN-γ overexpression. Thus, IL-10 might inhibit the effective clearance of *Leptospira* through the early regulation of particular inflammatory cytokines, leading to the persistence of bacteria and allowing chronic carriage in kidneys of resistant animals. It is noteworthy that no clinical signs were observed in *Leptospira*-infected Il-$10^{-/-}$ mice (Devlin et al., 2017) while IL-10 neutralization led to weight loss in infected OF1 mice (Matsui et al., 2017).

Finally, expression of the anti-inflammatory TGF-β is not modified in the blood (Vernel-Pauillac and Goarant, 2010; Fujita et al., 2015) while induced in the kidneys (Lowanitchapat et al., 2010) from *Leptospira*-infected hamsters during the acute stage of the disease. Interestingly, renal TGF-β1 expression is downregulated in asymptomatic mice during chronic carriage of *Leptospira* (Matsui et al., 2016) contrasting with unchanged expression level in fibrotic kidneys from susceptible mice compared to control (Fanton d'Andon et al., 2014; Ferrer et al., 2014). Consistently with the pro-fibrotic role of this cytokine in renal fibrogenesis (Higgins et al., 2018), and beyond its anti-inflammatory effect, this cytokine might specifically participate in renal pathophysiology of leptospirosis.

DISCUSSION

Inflammation is essential for the resolution of microbial infections and involves complex processes that finely coordinate cytokine production. Dysregulation of these mechanisms can trigger cytokine storm and related multi-organ failures as observed during severe leptospirosis and sepsis. Interestingly, the Jarisch-Herxheimer Reaction (JHR) noticed among leptospirosis patients is also characterized by large cytokine overproduction induced by massive bacterial product release consequent to antibiotic treatment (Friedland and Warrell, 1991; Guerrier and D'ortenzio, 2013; Guerrier et al., 2017). It is worth noticing that anti-TNF-α antibody therapy was proposed to prevent and ameliorate the JHR (Pound and May, 2005; Butler, 2017). However, despite the well-established involvement of cytokines in sepsis as observed in severe leptospirosis, no efficient treatment targeting inflammation was clinically validated. Indeed, data obtained from calibrated pre-clinical models can barely be translated to human diseases (van der Worp et al., 2010; McCarron et al., 2015). Thus, improving knowledge of the host innate immunity in leptospirosis is a major challenge to ameliorate therapeutic approaches, especially in severe forms of leptospirosis that are associated with a dramatic increase in mortality rate.

During *Leptospira* infection, inflammatory mediators are rapidly regulated in resistant models contrasting with awry production in susceptible hosts, and cytokines were thus proposed as promising biomarkers of the disease outcome in several clinical studies. However, larger and multi-factorial investigations are still required, including cytokine kinetics critical over the course of infection. Unfortunately, in patients, precise time of infection and infecting dose are hardly determined. Moreover, mediators are usually quantified in sera, but specific profiling of host response, including the identification of cellular sources for their production, in altered tissues would necessarily improve our understanding of inflammatory dysfunctions in organs.

Management of severe leptospirosis is also complex due to the diversity of clinical symptoms and immunological profiles found among patients. Human genetic background should be taken into consideration as highlighted by susceptible TLR4 deficient C3H/HeJ mice. However, only a few studies have investigated the correlation between genetic variability and leptospirosis severity and results are still under debate (Fialho et al., 2009; Esteves et al., 2014; Cedola et al., 2015). Finally, pathogen virulence will inevitably influence the host immune response. Several leptospiral components were described as virulence factors, including genes implicated in motility and LPS synthesis, but also genes whose functions still remain undescribed.

AUTHOR CONTRIBUTIONS

MM drafted the review. JC, SV, and MM contributed to the writing process.

FUNDING

The research position of MM and JC are financed by the Government of New Caledonia. This program is co-funded by the French Ministry of Research and Technology, Institut Pasteur de Nouvelle-Calédonie, and Institut Pasteur de Paris.

ACKNOWLEDGMENTS

The French Ministry of Research and Technology, Institut Pasteur in New Caledonia, and Institut Pasteur in Paris supported this work. Positions of MM and JC are financed by the Government of New Caledonia.

REFERENCES

Adler, B., and de la Pena Moctezuma, A. (2010). *Leptospira* and leptospirosis. *Vet. Microbiol.* 140, 287–296. doi: 10.1016/j.vetmic.2009.03.012

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124, 783–801. doi: 10.1016/j.cell.2006.02.015

Andrade, L., Rodrigues, A. C. Jr., Sanches, T. R., Souza, R. B., and Seguro, A. C. (2007). Leptospirosis leads to dysregulation of sodium transporters in the kidney and lung. *Am. J. Physiol. Renal Physiol.* 292, F586–F592. doi: 10.1152/ajprenal.00102.2006

Brown, J. P., Zachary, J. F., Teuscher, C., Weis, J. J., and Wooten, R. M. (1999). Dual role of interleukin-10 in murine Lyme disease: regulation of arthritis severity and host defense. *Infect. Immun.* 67, 5142–5150.

Butler, T. (2017). The jarisch-herxheimer reaction after antibiotic treatment of spirochetal infections: a review of recent cases and our understanding of pathogenesis. *Am. J. Trop. Med. Hyg.* 96, 46–52. doi: 10.4269/ajtmh.16-0434

Cedola, M., Chiani, Y., Pretre, G., Alberdi, L., Vanasco, B., and Gomez, R. M. (2015). Association of toll-like receptor 2 Arg753Gln and toll-like receptor 1 Ile602Ser single-nucleotide polymorphisms with leptospirosis in an argentine population. *Acta Trop.* 46, 73–80. doi: 10.1016/j.actatropica.2015.03.007

Chirathaworn, C., Supputtamongkol, Y., Lertmaharit, S., and Poovorawan, Y. (2016). Cytokine levels as biomarkers for leptospirosis patients. *Cytokine* 85, 80–82. doi: 10.1016/j.cyto.2016.06.007

Chousterman, B. G., Swirski, F. K., and Weber, G. F. (2017). Cytokine storm and sepsis disease pathogenesis. *Semin. Immunopathol.* 39, 517–528. doi: 10.1007/s00281-017-0639-8

Cohen, J. (2002). The immunopathogenesis of sepsis. *Nature* 420, 885–891. doi: 10.1038/nature01326

Correa-Rotter, R., Wesseling, C., and Johnson, R. J. (2014). CKD of unknown origin in Central America: the case for a Mesoamerican nephropathy. *Am. J. Kidney Dis.* 63, 506–520. doi: 10.1053/j.ajkd.2013.10.062

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015a). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

Costa, F., Wunder, E. A. Jr., De Oliveira, D., Bisht, V., Rodrigues, G., Reis, M. G., et al. (2015b). Patterns in *Leptospira* shedding in norway rats (*Rattus norvegicus*) from Brazilian slum communities at high risk of disease transmission. *PLoS Negl. Trop. Dis.* 9:e0003819. doi: 10.1371/journal.pntd.0003819

da Silva, J. B., Carvalho, E., Covarrubias, A. E., Ching, A. T., Mattaraia, V. G., Paiva, D., et al. (2012). Induction of TNF-alfa and CXCL-2 mRNAs in different organs of mice infected with pathogenic *Leptospira*. *Microb. Pathog.* 52, 206–216. doi: 10.1016/j.micpath.2012.01.002

Del Carlo Bernardi, F., Ctenas, B., Da Silva, L. F., Nicodemo, A. C., Saldiva, P. H., Dolhnikoff, M., et al. (2012). Immune receptors and adhesion molecules in human pulmonary leptospirosis. *Hum. Pathol.* 43, 1601–1610. doi: 10.1016/j.humpath.2011.11.017

Devlin, A. A., Halvorsen, P. J., Miller, J. C., and Laster, S. M. (2017). Il-10 deficient mice express IFN-gamma mRNA and clear *Leptospira interrogans* from their kidneys more rapidly than normal C57BL/6 mice. *Immunobiology* 222, 768–777. doi: 10.1016/j.imbio.2017.02.004

Dinarello, C. A. (2007). Historical review of cytokines. *Eur. J. Immunol.* 37(Suppl. 1), S34–S45. doi: 10.1002/eji.200737772

Domingos, R. H., Pavanel, E. B., Nakajima, E., Schons-Fonseca, L., Da Costa, R. M. A., De Franco, M., et al. (2017). Resistance of mice to *Leptospira* infection and correlation with chemokine response. *Immunobiology* 222, 1004–1013. doi: 10.1016/j.imbio.2017.05.017

Duell, B. L., Tan, C. K., Carey, A. J., Wu, F., Cripps, A. W., and Ulett, G. C. (2012). Recent insights into microbial triggers of interleukin-10 production in the host and the impact on infectious disease pathogenesis. *FEMS Immunol. Med. Microbiol.* 64, 295–313. doi: 10.1111/j.1574-695X.2012.00931.x

Estavoyer, J. M., Racadot, E., Couetdic, G., Leroy, J., and Grosperrin, L. (1991). Tumor necrosis factor in patients with leptospirosis. *Rev. Infect. Dis.* 13, 1245–1246. doi: 10.1093/clinids/13.6.1245

Esteves, L. M., Bulhões, S. M., Branco, C. C., Mota, F. M., Paiva, C., Cabral, R., et al. (2014). Human leptospirosis: seroreactivity and genetic susceptibility in the population of Sao Miguel Island (Azores, Portugal). *PLoS ONE* 9:e108534. doi: 10.1371/journal.pone.0108534

Fanton d'Andon, M., Quellard, N., Fernandez, B., Ratet, G., Lacroix-Lamandé, S., Vandewalle, A., et al. (2014). *Leptospira interrogans* induces fibrosis in the mouse kidney through Inos-dependent, TLR- and NLR-independent signaling pathways. *PLoS Negl. Trop. Dis.* 8:e2664. doi: 10.1371/journal.pntd.0002664

Ferrer, M. F., Scharrig, E., Alberdi, L., Cedola, M., Pretre, G., Drut, R., et al. (2014). Decay-accelerating factor 1 deficiency exacerbates leptospiral-induced murine chronic nephritis and renal fibrosis. *PLoS ONE* 9:e102860. doi: 10.1371/journal.pone.0102860

Fialho, R. N., Martins, L., Pinheiro, J. P., Bettencourt, B. F., Couto, A. R., Santos, M. R., et al. (2009). Role of HLA, KIR and cytokine gene polymorphisms in leptospirosis. *Hum. Immunol.* 70, 915–920. doi: 10.1016/j.humimm.2009.08.007

Friedland, J. S., and Warrell, D. A. (1991). The Jarisch-herxheimer reaction in leptospirosis: possible pathogenesis and review. *Rev. Infect. Dis.* 13, 207–210. doi: 10.1093/clinids/13.2.207

Fujita, R., Koizumi, N., Sugiyama, H., Tomizawa, R., Sato, R., and Ohnishi, M. (2015). Comparison of bacterial burden and cytokine gene expression in golden hamsters in early phase of infection with two different strains of *Leptospira interrogans*. *PLoS ONE* 10:e0132694. doi: 10.1371/journal.pone.0132694

Gautam, A., Dixit, S., Philipp, M. T., Singh, S. R., Morici, L. A., Kaushal, D., et al. (2011). Interleukin-10 alters effector functions of multiple genes induced by *Borrelia burgdorferi* in macrophages to regulate Lyme disease inflammation. *Infect. Immun.* 79, 4876–4892. doi: 10.1128/IAI.05451-11

Gogos, C. A., Drosou, E., Bassaris, H. P., and Skoutelis, A. (2000). Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J. Infect. Dis.* 181, 176–180. doi: 10.1086/315214

Gomes-Solecki, M., Santecchia, I., and Werts, C. (2017). Animal models of leptospirosis: of Mice and Hamsters. *Front. Immunol.* 8:58. doi: 10.3389/fimmu.2017.00058

Guerrier, G., and D'ortenzio, E. (2013). The jarisch-herxheimer reaction in leptospirosis: a systematic review. *PLoS ONE* 8:e59266. doi: 10.1371/journal.pone.0059266

Guerrier, G., Lefèvre, P., Chouvin, C., and D'ortenzio, E. (2017). Jarisch-herxheimer reaction among patients with leptospirosis: incidence and risk factors. *Am. J. Trop. Med. Hyg.* 96, 791–794. doi: 10.4269/ajtmh.16-0457

Haake, D. A., and Levett, P. N. (2015). Leptospirosis in humans. *Curr. Top. Microbiol. Immunol.* 387, 65–97. doi: 10.1007/978-3-662-45059-8_5

Herath, N. J., Kularatne, S. A., Weerakoon, K. G., Wazil, A., Subasinghe, N., and Ratnatunga, N. V. (2014). Long term outcome of acute kidney injury due to leptospirosis? A longitudinal study in Sri Lanka. *BMC Res. Notes* 7:398. doi: 10.1186/1756-0500-7-398

Higgins, S. P., Tang, Y., Higgins, C. E., Mian, B., Zhang, W., Czekay, R. P., et al. (2018). TGF-beta1/p53 signaling in renal fibrogenesis. *Cell Signal* 43, 1–10. doi: 10.1016/j.cellsig.2017.11.005

Jin, D., Ojcius, D. M., Sun, D., Dong, H., Luo, Y., Mao, Y., et al. (2009). *Leptospira interrogans* induces apoptosis in macrophages via caspase-8- and caspase-3-dependent pathways. *Infect. Immun.* 77, 799–809. doi: 10.1128/IAI.00914-08

Kyriakidis, I., Samara, P., and Papa, A. (2011). Serum TNF-alpha, sTNFR1, IL-6, IL-8 and IL-10 levels in Weil's syndrome. *Cytokine* 54, 117–120. doi: 10.1016/j.cyto.2011.01.014

Levett, P. N. (2001). Leptospirosis. *Clin. Microbiol. Rev.* 14, 296–326. doi: 10.1128/CMR.14.2.296-326.2001

Lingappa, J., Kuffner, T., Tappero, J., Whitworth, W., Mize, A., Kaiser, R., et al. (2004). HLA-DQ6 and ingestion of contaminated water: possible gene-environment interaction in an outbreak of Leptospirosis. *Genes Immun.* 5, 197–202. doi: 10.1038/sj.gene.6364058

Lowanitchapat, A., Payungporn, S., Seeremaspun, A., Ekpo, P., Phulsuksombati, D., Poovorawan, Y., et al. (2010). Expression of TNF-alpha, TGF-beta, IP-10 and IL-10 mRNA in kidneys of hamsters infected with pathogenic *Leptospira*. *Comp. Immunol. Microbiol. Infect. Dis.* 33, 423–434. doi: 10.1016/j.cimid.2009.05.001

Marinho, M., Taparo, C. V., Oliveira-Junior, I. S., Perri, S. H., and Cardoso, T. C. (2015). Tissue apoptosis in mice infected with *Leptospira interrogans* serovar Icterohaemorrhagiae. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 21:22. doi: 10.1186/s40409-015-0022-y

Matsui, M., Roche, L., Geroult, S., Soupe-Gilbert, M. E., Monchy, D., Huerre, M., et al. (2016). Cytokine and chemokine expression in kidneys during chronic leptospirosis in reservoir and susceptible animal models. *PLoS ONE* 11:e0156084. doi: 10.1371/journal.pone.0156084

Matsui, M., Roche, L., Soupé-Gilbert, M.-E., Hasan, M., Monchy, D., and Goarant, C. (2017). High level of IL-10 expression in the blood of animal models possibly relates to resistance against leptospirosis. *Cytokine* 96, 144–151. doi: 10.1016/j.cyto.2017.03.009

Matsui, M., Rouleau, V., Bruyere-Ostells, L., and Goarant, C. (2011). Gene expression profiles of immune mediators and histopathological findings in animal models of leptospirosis: comparison between susceptible hamsters and resistant mice. *Infect. Immun.* 79, 4480–4492. doi: 10.1128/IAI.05727-11

McCarron, E. P., Williams, D. P., Antoine, D. J., Kipar, A., Lemm, J., Stehr, S., et al. (2015). Exploring the translational disconnect between the murine and human inflammatory response: analysis of LPS dose–response relationship in murine versus human cell lines and implications for translation into murine models of sepsis. *J. Inflamm. Res.* 8, 201–209. doi: 10.2147/JIR.S89097

Mikulski, M., Boisier, P., Lacassin, F., Soupe-Gilbert, M. E., Mauron, C., Bruyere-Ostells, L., et al. (2015). Severity markers in severe leptospirosis: a cohort study. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 687–695. doi: 10.1007/s10096-014-2275-8

Nahori, M. A., Fournie-Amazouz, E., Que-Gewirth, N. S., Balloy, V., Chignard, M., Raetz, C. R., et al. (2005). Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. *J. Immunol.* 175, 6022–6031. doi: 10.4049/jimmunol.175.9.6022

Opal, S. M., and Depalo, V. A. (2000). Anti-inflammatory cytokines. *Chest* 117, 1162–1172. doi: 10.1378/chest.117.4.1162

Papa, A., and Kotrotsiou, T. (2015). Cytokines in human leptospirosis. *Trans. R. Soc. Trop. Med. Hyg.* 109, 749–754. doi: 10.1093/trstmh/trv095

Picardeau, M. (2015). Leptospirosis: updating the global picture of an emerging neglected disease. *PLoS Negl. Trop. Dis.* 9:e0004039. doi: 10.1371/journal.pntd.0004039

Pound, M. W., and May, D. B. (2005). Proposed mechanisms and preventative options of Jarisch-Herxheimer reactions. *J. Clin. Pharm. Ther.* 30, 291–295. doi: 10.1111/j.1365-2710.2005.00631.x

Reis, E. A., Hagan, J. E., Ribeiro, G. S., Teixeira-Carvalho, A., Martins-Filho, O. A., Montgomery, R. R., et al. (2013). Cytokine response signatures in disease progression and development of severe clinical outcomes for leptospirosis. *PLoS Negl. Trop. Dis.* 7:e2457. doi: 10.1371/journal.pntd.0002457

Ricciotti, E., and FitzGerald, G. A. (2011). Prostaglandins and inflammation. *Arterioscler. Thromb. Vasc. Biol.* 31, 986–1000. doi: 10.1161/ATVBAHA.110.207449

Richer, L., Potula, H. H., Melo, R., Vieira, A., and Gomes-Solecki, M. (2015). Mouse model for sublethal *Leptospira interrogans* infection. *Infect. Immun.* 83, 4693–4700. doi: 10.1128/IAI.01115-15

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832. doi: 10.1016/j.cell.2010.01.040

Schulte, W., Bernhagen, J., and Bucala, R. (2013). Cytokines in sepsis: potent immunoregulators and potential therapeutic targets–an updated view. *Mediators Inflamm.* 2013:165974. doi: 10.1155/2013/165974

Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., et al. (2016). The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA* 315, 801–810. doi: 10.1001/jama.2016.0287

Song, G. Y., Chung, C. S., Chaudry, I. H., and Ayala, A. (1999). What is the role of interleukin 10 in polymicrobial sepsis: anti-inflammatory agent or immunosuppressant? *Surgery* 126, 378–383. doi: 10.1016/S0039-6060(99)70180-7

Sullivan, J. P., Nair, N., Potula, H. H., and Gomes-Solecki, M. (2017). Eye-drop inoculation leads to sublethal leptospirosis in mice. *Infect. Immun.* 85:e01050-16. doi: 10.1128/IAI.01050-16

Tajiki, M. H., Nakama, A. S. Y., and Salomão, R. (1997). The ratio of plasma levels Of Il-10/Tnf-alpha and its relationship to disease severity and survival in patients with leptospirosis. *Braz. J. Infect. Dis.* 1, 138–141.

Tajiki, M. H., and Salomão, R. (1996). Association of plasma levels of Tumour Necrosis Factor α with severity of disease and mortality among patients with leptospirosis. *Clin. Infect. Dis.* 23, 1177–1178. doi: 10.1093/clinids/23.5.1177

Tisoncik, J. R., Korth, M. J., Simmons, C. P., Farrar, J., Martin, T. R., and Katze, M. G. (2012). Into the eye of the cytokine storm. *Microbiol. Mol. Biol. Rev.* 76, 16–32. doi: 10.1128/MMBR.05015-11

Trevejo, R. T., Rigau-Perez, J. G., Ashford, D. A., Mcclure, E. M., Jarquin-Gonzalez, C., Amador, J. J., et al. (1998). Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua 1995. *J. Infect. Dis.* 178, 1457–1463. doi: 10.1086/314424

Turner, M. D., Nedjai, B., Hurst, T., and Pennington, D. J. (2014). Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim. Biophys. Acta* 1843, 2563–2582. doi: 10.1016/j.bbamcr.2014.05.014

van der Worp, H. B., Howells, D. W., Sena, E. S., Porritt, M. J., Rewell, S., O'collins, V., et al. (2010). Can animal models of disease reliably inform human studies? *PLoS Med.* 7:e1000245. doi: 10.1371/journal.pmed.1000245

Vernel-Pauillac, F., and Goarant, C. (2010). Differential cytokine gene expression according to outcome in a hamster model of leptospirosis. *PLoS Negl. Trop. Dis.* 4:e582. doi: 10.1371/journal.pntd.0000582

Vernel-Pauillac, F., and Merien, F. (2006). Proinflammatory and immunomodulatory cytokine mRNA time course profiles in hamsters infected with a virulent variant of *Leptospira interrogans*. *Infect. Immun.* 74, 4172–4179. doi: 10.1128/IAI.00447-06

Volz, M. S., Moos, V., Allers, K., Luge, E., Mayer-Scholl, A., Nockler, K., et al. (2015). Specific CD4+ T-cell reactivity and cytokine release in different clinical presentations of leptospirosis. *Clin. Vaccine Immunol.* 22, 1276–1284. doi: 10.1128/CVI.00397-15

Wagenaar, J. F., Gasem, M. H., Goris, M. G., Leeflang, M., Hartskeerl, R. A., Van Der Poll, T., et al. (2009a). Soluble ST2 levels are associated with bleeding in patients with severe leptospirosis. *PLoS Negl. Trop. Dis.* 3:e453. doi: 10.1371/journal.pntd.0000453

Wagenaar, J. F., Goris, M. G., Gasem, M. H., Isbandrio, B., Moalli, F., Mantovani, A., et al. (2009b). Long pentraxin PTX3 is associated with mortality and disease severity in severe Leptospirosis. *J. Infect.* 58, 425–432. doi: 10.1016/j.jinf.2009.04.004

Wagenaar, J. F., Goris, M. G., Partiningrum, D. L., Isbandrio, B., Hartskeerl, R. A., Brandjes, D. P., et al. (2010). Coagulation disorders in patients with severe leptospirosis are associated with severe bleeding and mortality. *Trop. Med. Int. Health* 15, 152–159. doi: 10.1111/j.1365-3156.2009.02434.x

Wang, L., Du, F., and Wang, X. (2008). TNF-a induces two distinct caspase-8 activation pathways. *Cell* 133, 693–703. doi: 10.1016/j.cell.2008.03.036

Werts, C. (2017). "Interaction of leptospira with the innate immune system," in *Current Topics in Microbiology and Immunology* (Berlin; Heidelberg: Springer).

Wink, D. A., Hines, H. B., Cheng, R. Y., Switzer, C. H., Flores-Santana, W., Vitek, M. P., et al. (2011). Nitric oxide and redox mechanisms in the immune response. *J. Leukoc. Biol.* 89, 873–891. doi: 10.1189/jlb.1010550

Wynn, T. A. (2015). Type 2 cytokines: mechanisms and therapeutic strategies. *Nat. Rev. Immunol.* 15, 271–282. doi: 10.1038/nri3831

Yamagata, T., Yamagata, Y., Nishimoto, T., Hirano, T., Nakanishi, M., Minakata, Y., et al. (2009). The regulation of amiloride-sensitive epithelial sodium channels by tumor necrosis factor-alpha in injured lungs and alveolar type II cells. *Respir. Physiol. Neurobiol.* 166, 16–23. doi: 10.1016/j.resp.2008.12.008

Yilmaz, H., Turhan, V., Yasar, K. K., Hatipoglu, M., Sunbul, M., and Leblebicioglu, H. (2015). Characteristics of leptospirosis with systemic inflammatory response syndrome: a multicenter study. *Ann. Clin. Microbiol. Antimicrob.* 14:54. doi: 10.1186/s12941-015-0117-x

Zeng, L., Gu, W., Chen, K., Jiang, D., Zhang, L., Du, D., et al. (2009). Clinical relevance of the interleukin 10 promoter polymorphisms in Chinese Han patients with major trauma: genetic association studies. *Crit. Care* 13:R188. doi: 10.1186/cc8182

Zhao, H. Q., Li, W. M., Lu, Z. Q., Sheng, Z. Y., and Yao, Y. M. (2015). The growing spectrum of anti-inflammatory interleukins and their potential roles in the development of sepsis. *J. Interferon Cytokine Res.* 35, 242–251. doi: 10.1089/jir.2014.0119

Zhao, X., Bausano, B., Pike, B. R., Newcomb-Fernandez, J. K., Wang, K. K., Shohami, E., et al. (2001). TNF-alpha stimulates caspase-3 activation and apoptotic cell death in primary septo-hippocampal cultures. *J. Neurosci. Res.* 64, 121–131. doi: 10.1002/jnr.1059

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 08 March 2018
doi: 10.3389/fcimb.2018.00063

Role of Murine Complement Component C5 in Acute *in Vivo* Infection by Pathogenic *Leptospira interrogans*

Íris A. de Castro[1†], Lorena Bavia[1†], Tatiana R. Fraga[1], Mariane T. Amano[1], Leandro C. D. Breda[1], Adriana P. Granados-Martinez[1], Ana M. G. da Silva[2], Silvio A. Vasconcellos[3] and Lourdes Isaac[1]*

[1] Laboratory of Complement, Department of Immunology, University of São Paulo, São Paulo, Brazil, [2] Institute of Tropical Medicine, University of São Paulo, São Paulo, Brazil, [3] Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Nadia Benaroudj,
Institut Pasteur, France

Reviewed by:
Alan John Alexander McBride,
Universidade Federal de Pelotas, Brazil
Ricardo Martin Gomez,
Instituto de Biotecnologia y Biologia Molecular, CONICET-UNLP, Argentina
Marcelo Hill,
Institut Pasteur de Montevideo, Uruguay

***Correspondence:**
Lourdes Isaac
louisaac@icb.usp.br

[†] These authors have contributed equally to this work.

Received: 21 September 2017
Accepted: 13 February 2018
Published: 08 March 2018

Citation:
de Castro ÍA, Bavia L, Fraga TR, Amano MT, Breda LCD, Granados-Martinez AP, da Silva AMG, Vasconcellos SA and Isaac L (2018) Role of Murine Complement Component C5 in Acute in Vivo Infection by Pathogenic Leptospira interrogans. Front. Cell. Infect. Microbiol. 8:63. doi: 10.3389/fcimb.2018.00063

Leptospirosis is considered one of the most important zoonosis worldwide. The activation of the Complement System is important to control dissemination of several pathogens in the host. Only a few studies have employed murine models to investigate leptospiral infection and our aim in this work was to investigate the role of murine C5 during *in vivo* infection, comparing wild type C57BL/6 (B6 $C5^{+/+}$) and congenic C57BL/6 (B6 $C5^{-/-}$, C5 deficient) mice during the first days of infection. All animals from both groups survived for at least 8 days post-infection with pathogenic *Leptospira interrogans* serovar Kennewicki strain Fromm (LPF). At the third day of infection, we observed greater numbers of LPF in the liver of B6 $C5^{-/-}$ mice when compared to B6 $C5^{+/+}$ mice. Later, on the sixth day of infection, the LPF population fell to undetectable levels in the livers of both groups of mice. On the third day, the inflammatory score was higher in the liver of B6 $C5^{+/+}$ mice than in B6 $C5^{-/-}$ mice, and returned to normal on the sixth day of infection in both groups. No significant histopathological differences were observed in the lung, kidney and spleen from both infected B6 $C5^{+/+}$ than B6 $C5^{-/-}$ mice. Likewise, the total number of circulating leukocytes was not affected by the absence of C5. The liver levels of IL-10 on the sixth day of infection was lower in the absence of C5 when compared to wild type mice. No significant differences were observed in the levels of several inflammatory cytokines when B6 $C5^{+/+}$ and B6 $C5^{-/-}$ were compared. In conclusion, C5 may contribute to the direct killing of LPF in the first days of infection in C57BL/6 mice. On the other hand, other effector immune mechanisms probably compensate Complement impairment since the mice survival was not affected by the absence of C5 and its activated fragments, at least in the early stage of this infection.

Keywords: Complement System, C5, *Leptospira*, leptospirosis, murine model, inflammation

INTRODUCTION

Leptospirosis is an emerging disease worldwide which affects approximately one million patients each year (Costa et al., 2015), mainly in developing countries with tropical and subtropical climates and underdeveloped waste and sewage management systems. For some patients, this disease can be asymptomatic, while others may present symptoms ranging from a mild infection to the development of fever, jaundice, liver and kidney failure and lung hemorrhage, resulting in fatality rates higher than 5–10% (Torgerson et al., 2015). Infection results from the contact of injured skin or mucosa with soil and/or water contaminated with leptospires released by the urine of infected animals (Ko et al., 2009). Rodents are considered asymptomatic to this pathogen and they represent the main transmission source especially in urban centers (Adler, 2015).

Since *Leptospira* spp. is considered an extracellular pathogen, the activation of the Complement System (CS), phagocytosis and production of specific antibodies play an important role in controlling this infection (reviewed by Fraga et al., 2016). The CS is necessary to control proliferation and dissemination of several microorganisms in the host which is clearly confirmed by the higher susceptibility to infections observed in C5 deficient patients (Aguilar-Ramirez et al., 2009) or in patients deficient of other CS proteins (Macedo and Isaac, 2016). CS can be activated by the Classic, Alternative and/or Lectin Pathways, and all three converge to a common terminal activation pathway which leads to lysis caused by the formation of the membrane attack complex (MAC) on the microorganism surface. The terminal pathway depends on the formation of C5 convertase enzymes which cleave C5 in two fragments. C5a, the smaller fragment, is an important anaphylatoxin involved in mast cell and basophil degranulation which releases histamine and other inflammatory mediators like prostaglandins and leukotrienes (Guo and Ward, 2005). C5a is also a well-known chemoattractant factor for neutrophils, monocytes and eosinophils during acute inflammation. C5b, the larger fragment, is the first to participate in MAC ($C5b6789_n$) formation (Podack et al., 1984; Serna et al., 2016). Besides contributing to control systemic or local infection, the inflammatory properties observed during C5 activation and the participation of receptors such as C5aR1 may be responsible for local tissue damage (Ward, 2010). The ability to induce cellular lysis and the synergistic interactions with other immune mechanisms highlight the importance of the CS in mounting a robust immune response.

Nonpathogenic leptospires are rapidly killed *in vitro* after CS activation while pathogenic species such as *Leptospira interrogans* serovar Kennewicki strain Fromm (LPF) are resistant. LPF immune evasion mechanisms include: (i) binding to host CS regulatory proteins Factor H (Meri et al., 2005), C4b binding protein (Barbosa et al., 2009, 2010; Breda et al., 2015) and vitronectin (da Silva et al., 2015); (ii) binding to host proteases such as plasminogen (Vieira et al., 2010, 2011; Castiblanco-Valencia et al., 2016) which once converted to its active form, plasmin, may cleave CS proteins; and (iii) secretion of leptospiral proteases that cleave Complement proteins (Fraga et al., 2014; Amamura et al., 2017).

Even though mice are considered asymptomatic to *Leptospira* infection, the possibility of using congenic mouse models allows researchers to investigate in more depth important questions related to the pathophysiology of the immune responses. In addition, mice are considered good models to study sub-lethal infection and chronic colonization often observed in leptospirosis patients (reviewed by Gomes-Solecki et al., 2017). To date, only one study has investigated the importance of the CS during infection by *Leptospira* spp. in a murine model. Ferrer et al. (2014) studied the relevance of decay accelerating factor (DAF, CD55) at 14 and 90 days after infection. DAF is an important regulatory membrane protein that protects host cells from autologous CS activation by binding to membrane-bound C3b and inhibiting the formation and accelerating the decay of C3-convertases and C5-convertases (Nicholson-Weller et al., 1981, 1983). However, differently from other regulatory proteins like Factor H and Factor I, DAF does not modulate serum levels of C3 and is not related directly to the elimination of microorganisms. Ferrer et al. (2014) observed that C57BL/6J Daf $1^{-/-}$ mice infected with *L. interrogans* serovar Copenhageni presented higher numbers of leptospires in the kidney 14 days post-infection when compared to wild type mice and the lack of DAF was associated with chronic nephritis and renal fibrosis. These symptoms are probably related to persistent injury caused by uncontrolled CS activation on proximal renal tubules.

To evaluate the importance of the component C5 in the control of *in vivo* leptospiral infection, we infected C57BL/6 (B6) wild type (B6 $C5^{+/+}$) and congenic C57BL/6 C5 deficient mice (B6 $C5^{-/-}$) with pathogenic *L. interrogans* serovar Kennewick type Pomona Fromm and analyzed several aspects of the immune response during the first days of infection.

MATERIALS AND METHODS

Leptospira Cultures

Pathogenic *L. interrogans* serovar Kennewicki, strain Pomona Fromm (LPF) and saprophytic *L. biflexa* strain Patoc I (Patoc) were obtained from the Laboratory of Bacterial Zoonosis at the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo. Leptospires were kept under aerobic conditions at 29°C for 5–7 days in Ellinghausen McCullough Johnson and Harris culture medium (EMJH) supplemented with 10% inactivated rabbit serum, L-asparagine (0.015%), sodium pyruvate (0.001%), calcium chloride (0.001%), magnesium chloride (0.001%), peptone (0.03%), and meat extract (0.02%).

Mice Infection With LPF

We used C57BL/6 C5 normal (B6 $C5^{+/+}$) mice and the corresponding congenic C57BL/6 C5 deficient (B6 $C5^{-/-}$) strain, generated in our laboratory (Bavia et al., 2014). We also included another C5 deficient (A/J) mouse strain to evaluate the survival during LPF infection. All mice were 4–5 weeks old males obtained from the Animal Facility of the Department of Immunology, Institute of Biomedical Sciences from the University of São Paulo. Male mice were used to avoid interference by sexual hormones and since they present higher levels of Complement System activity and higher serum levels of

C6 and C9 from the terminal pathway (Kotimaa et al., 2016). Mice were intraperitoneally infected with 1.5×10^8 LPF in phosphate buffered saline pH 7.4 (PBS) and euthanized on the third or sixth days after infection. Control groups were inoculated with sterile PBS and euthanized 6 days post inoculation. We selected these days of infection based on previous work (da Silva et al., 2012). Mice were previously anesthetized with ketamine and xylazine (100 and 10 mg/kg, respectively) before manipulation. Hamsters, a susceptible experimental animal, were used to confirm the virulence by infecting them with LPF culture. Three to five days post-inoculation of LPF, infected hamster presented jaundice, photo sensibility, uveitis, weight loss and prostration. This work was carried out as approved by the Ethics Committee on Animal Experimentation (Certificate 061/10/CEEA). The number of mice used in each experiment is indicated in each figure legend.

DNA Extraction From Liver and *Leptospira* DNA Quantification by qPCR

Total DNA was extracted from 20 to 25 mg of liver from LPF-infected or control (PBS) mice using the Illustra Tissue & Cells Genomic Prep Mini Spin kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) following the manufacturer's instructions. DNA concentration and purity were measured using a Nanodrop nd-1000 spectrophotometer (Thermo Fisher Scientific).

To determine the leptospiral load in the liver from infected mice, we employed quantitative PCR (qPCR) using 96 well microtiter plates (Life Technologies). A concentration of liver DNA was adjusted to 50 ng/μL. The standard curve was prepared using DNA extracted from 10-fold dilutions from 10^8 to 10^1 heat-killed *L. interrogans*. We used 1 μL for the standard curve or of samples followed by 20 pmol/μL of both primers complementary to the *Leptospira 16S rRNA* gene (forward primer: 5′-TAGTGAACGGGATAGATAC-3′; reverse primer 5′-GGTCTACTT AATCCGTTAGG-3′) and 10 μL SYBR Green master mix (Life Technologies) in a final volume of 20 μL. Samples were amplified in a thermocycler Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following program: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by two cycles of 95°C for 15 s and 60°C for 1 min, and a final step at from 0.5 to 95° C (ramp) for 15 s.

Histopathological and Immunochemical Analyses

Liver, kidney, lung and spleen samples were fixed in formalin solution (3.7% formaldehyde in PBS pH 7.4). Microscopic slides were prepared with 5 μm tissue sections stained with hematoxylin-eosin (HE). To quantify the histopathological alterations in the liver, we considered the following criteria: (a) sinusoidal hypercellularity and presence of leucocyte infiltrates; (b) presence of mitotic cells; (c) hepatocyte destrabecullation; and (d) cell necrosis. Liver sections with only one of the above criteria were classified with a score of 1; with two of above criteria, given a score of 2; with three criteria, a score of 3 and with all the above criteria, a score of 4. To quantify the histopathological alterations in the lung, we considered the following criteria: (a) presence of nodular interstitial pneumonitis (IP) was classified with a score of 1; (b) presence of diffuse IP was classified with a score of 2. To quantify the histopathological alterations in the spleen, we considered the following criteria: (a) presence of perifollicular hyperplasia was classified with a score of 1; (b) presence of central follicular hyperplasia was classified with a score of 2; (c) presence of both perifollicular and central follicular hyperplasia was classified with a score of 3.

Immunohistochemistry analyses were performed to assess the presence of leptospiral antigens in the organs. Liver, kidney, lung and spleen sections were deparaffinized and rehydrated. Tissue sections were then incubated with Target Retrieval solution (DAKO S1699) heated using a steamer to unmask antigen(s). The presence of endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 20 min at room temperature. Preparations were then incubated for 18 h at 4°C with rabbit polyclonal anti-*Leptospira* antibodies diluted at 1:19.000 in PBS supplemented with 0.1% BSA. After several washes with PBS, the tissue sections were incubated with secondary EnVision + System HRP labeled polymer anti-rabbit IgG (DAKO K 4002) for 30 min at room temperature. The slides were washed again and incubated with the chromogen Diamino-benzidine (DAB, Sigma Chemical Co. USA) in the presence of 2% H_2O_2 (10 vol.) for 5 min. After this procedure, the tissue sections were stained with hematoxylin.

Biochemical Assays

Hepatic damage was indirectly evaluated by measuring alanine transaminase and aspartate transaminase (AST) (Bioclin Quibasa, Belo Horizonte, MG, Brazil) serum concentrations, while kidney function was evaluated by urea) and uric acid (Bioclin Quibasa, Belo Horizonte, MG, Brazil) serum levels as described by Bavia et al. (2014).

Blood Leukocyte Counting

Fresh blood samples were obtained from orbital venous plexus with heparinized glass capillary tubes from anesthetized mice. Samples were diluted in Türk solution (4.76 mM acetic acid, 6.25 μM methylene blue) and total peripheral blood leukocytes were counted in a Neubauer chamber.

Cytokine Measurements

Liver levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, IL-12p40, and IL-12p70 were determined by ELISA as described in Bavia et al. (2015). Serum levels of IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), TNF-α, and IL-12p70 were determined using the Inflammation CBA kit (BD Bioscience, Franklin Lakes, New Jersey, United States), according to the manufacturer's instructions. Data acquisition was performed using a FACS Canto II flow cytometer and data analysis was performed using FCAP Array™ v3.0.1 Software (both from BD Bioscience, Franklin Lakes, New Jersey, United States).

Leptospires Killing Assay

LPF and Patoc cultures were centrifuged at 2,800 × *g* at 21°C for 20 min, resuspended in PBS and counted in a Petroff-Hausser chamber. A total of 1 x 10^8 leptospires was incubated for 2 h at 37°C with B6 $C5^{+/+}$ or B6 $C5^{-/-}$ serum (40% serum and 60% PBS, 200 μL). Normal human serum (NHS) was used as a positive control (Barbosa et al., 2009). Viable bacteria were counted using dark-field microscopy. To inactivate the CS in mice or human serum, they were previously heated at 56°C/30 min. The number of viable leptospires incubated with heat-inactivated serum was considered 100% survival.

Statistical Analysis

Leptospire killing assay was plotted considering the mean and standard deviation and analyzed by Mann-Whitney test. The other results were plotted with the mean and standard error for each group and submitted to ANOVA two-way with Tukey post-test. All analyses considered a significance level of at least 95% ($p < 0.05$).

RESULTS

LPF Is Detected Mainly in the Liver in the Early Days of Infection

On the third day post-infection, C5 deficient mice (B6 $C5^{-/-}$) were observed to carry a higher number of LPF in the liver when compared to C5-sufficient mice (B6 $C5^{+/+}$). However, on the sixth day post-infection, the presence of LPF was undetectable in both mice strains by qPCR (**Figure 1**). The presence of LPF antigens in the liver was also investigated by immunohistochemical analysis, but no differences were observed between B6 $C5^{+/+}$ or B6 $C5^{-/-}$ mice (**Supplementary Figure 1**). The survival of LPF infected mice was independent of C5 since all B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice survived when monitored up to eight days of infection with LPF**.** In addition, we used another C5 deficient mouse strain (A/J) with 10^3, 10^5, 10^7, and 10^9 LPF (minimal of 5 mice for each inoculum). Again, all mice survived up to 21 days of infection.

C5 Triggers More Inflammation in the Liver During Early Infection With LPF

Since C5a and its receptor C5aR1 are clearly associated with liver inflammation, tissue injury and regeneration (Strey et al., 2003; Markiewski et al., 2004, 2009), we compared livers from LPF-infected B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice. As illustrated in **Figure 2A,** leukocyte infiltration was observed on the third and sixth days of infection around the portal spaces and within the hepatic sinusoids. This infiltration was composed mainly of mononuclear cells and was more evident in the presence of C5 on the third day of infection. Mitotic cells were also present in B6 $C5^{+/+}$ mice on the third and sixth day, while in B6 $C5^{-/-}$ mitotic cells were found only on the sixth day. These observations suggest an intense inflammatory response accompanied by hepatocellular lesions by hepatocyte proliferation primarily in B6 $C5^{+/+}$ mice (**Figure 2A**). These hepatic changes were significantly more intense in B6 $C5^{+/+}$ mice than in B6 $C5^{-/-}$ on the third day of infection. On the sixth day of infection, C5 deficient mice continued to present fewer lesions than B6 $C5^{+/+}$ mice but no significant differences in the scores were observed between them (**Figure 2B**). To monitor liver damage, levels of ALT and AST enzymes were determined in the serum and no significant differences were observed in B6 $C5^{-/-}$ and B6 $C5^{+/+}$ mice (**Supplementary Figure 2**).

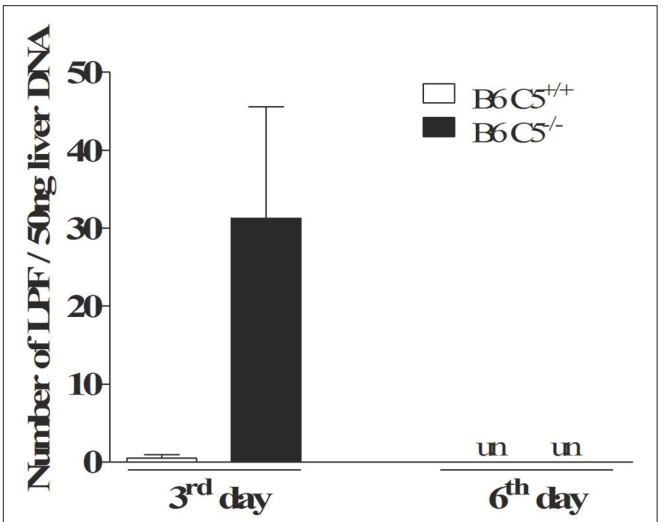


FIGURE 1 | Acute infection with leptospires in C5 deficient mice. Relative number of leptospires in mouse liver was quantified by qPCR. Infected B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice were euthanized after 3 and 6 days post-infection. Liver DNA was extracted and the relative number of leptospires was determined by qPCR after amplification of *16S rRNA* gene. un: undetectable.

Lung and Kidney of LPF Infected Mice

During human leptospirosis, lung and kidney may also be affected (Ko et al., 2009). Although LPF antigens were practically undetectable by immunohistochemical analysis in lung and kidney of both B6 $C5^{-/-}$ and B6 $C5^{+/+}$ mice on the third and sixth days post-infection (data not shown), in our model, LPF provoked lesions in lung, characterized by thickening of the alveolar septa accompanied by lymphocyte infiltration (**Supplementary Figure 3A).** However, no significant differences were observed between B6 $C5^{+/+}$ and B6 $C5^{-/-}$ infected mice (**Supplementary Figure 3B**). Moreover, no lesions were observed in the kidneys of B6 $C5^{+/+}$ and B6 $C5^{-/-}$ LPF infected mice (**Supplementary Figure 4A**). In addition, the serum concentrations of urea and uric acid were measured and both were altered at the sixth day of infection. However, these differences were not C5 dependent (**Supplementary Figure 4B**).

Blood Leukocyte Analysis

The total number of circulating leukocytes was determined in the blood of both B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice infected and non-infected with LPF. This number significantly decreased on the sixth day when compared to the third day of infection in both mouse strains infected with LPF (**Figure 3**). No significant difference could be attributed to the presence of C5 either on the third or the sixth days of infection.

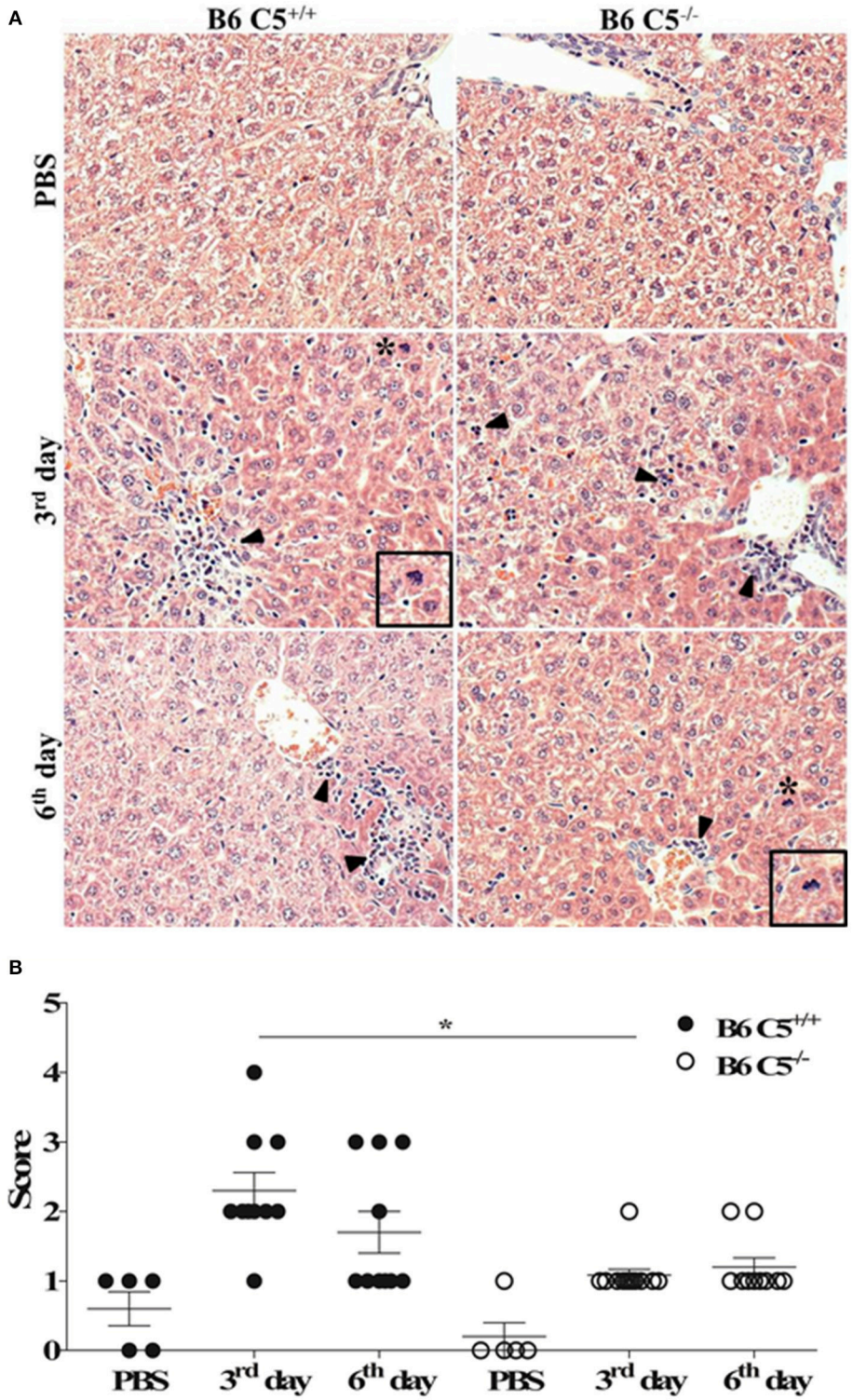


FIGURE 2 | Liver histopathological analyses in LPF infected mice. Mice were inoculated i/p with 1.5×10^8 LPF or only PBS and then euthanized on the third or the sixth day post-infection ($n \geq 5$). **(A)** Liver sections (3–5 μm) were stained with (HE) and evaluated at 200x magnification. Arrowheads indicate leukocyte infiltrates in the portal space and in the hepatic sinusoids. Asterisks indicate mitotic cells. Inset: mitotic cells in larger magnification. **(B)** Total scores of hepatic lesions. The significant difference ($p < 0.05$) is represented by *when B6 $C5^{+/+}$ were compared to B6 $C5^{-/-}$ mice.

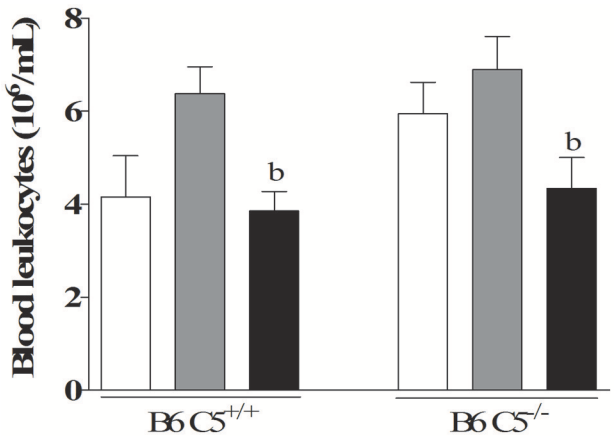


FIGURE 3 | Circulating leukocytes in LPF infected mice. Mice were inoculated i/p with 1.5×10^8 LPF ($n \geq 5$) and the total number of leukocytes was determined after 3 and 6 days post-infection using a hemocytometer chamber. b represents significant difference between the third and sixth days of infection.

Spleen Modifications During LPF Infection

Both mouse strains developed similar splenomegaly during infection by LPF (**Figure 4A**). Histopathological analysis indicated that white pulp is expanded after inoculation of LPF, followed by clonal expansion of lymphocytes (**Figures 4B,C**). On the third day of infection, we observed increased perifolicular activity, indicative of B lymphocyte proliferation. On the sixth day of infection T cell expansion in the centrofolicular region (periarteriolar lymphoid sheath) is more evident. However, no significant differences in the spleen parenchyma could be observed between B6 $C5^{+/+}$ and B6 $C5^{-/-}$ infected mice (**Figures 4B,C**). The presence of LPF antigens was observed in the spleen of all infected mice (**Figure 4D**), however no significant differences were observed in this organ at the third and sixth days of infection when B6 $C5^{+/+}$ and B6 $C5^{-/-}$ strains were compared.

Liver and Blood Cytokine Levels

In spite of a more intense inflammatory response observed in the presence of C5, no significant changes in the concentration of cytokines in the liver of infected mice were observed in B6 $C5^{+/+}$ mice when compared to B6 $C5^{-/-}$ mice (**Supplementary Figure 5**). The hepatic levels of TNF, IL-6, IL-1β, and IL-12p70 did not change significantly upon infection (**Supplementary Figures 5A,B,D,F**). B6 $C5^{-/-}$ mice inoculated with PBS presented a higher concentration of liver IL-12p40 than B6 $C5^{+/+}$. On the other hand, B6 $C5^{-/-}$ mice presented a lower concentration of liver IL-12p40 on the sixth day of infection when compared with PBS-treated mice (**Supplementary Figure 5E**). In the absence of C5, a tendency to lower IL-10 levels were observed on the sixth day of infection ($p = 0.055$) when compared to B6 $C5^{+/+}$ mice (**Supplementary Figure 5C**) at the same time. Blood levels of TNF-α, IFN-γ, and IL-6 were not significantly affected by LPF infection (**Supplementary Figures 5G–I**). The concentrations of MCP-1 (CCL2), IL-10, and IL-12p70 in the serum from both B6 $C5^{+/+}$ and B6 $C5^{+/+}$ mice were below detectable levels on the third and the sixth days post-infection.

LPF Is Resistant to CS Mediated Killing by Murine Serum

To investigate the possible role of C5 in murine resistance to LPF infection, cultures of this pathogenic leptospire were incubated *in vitro* with 40% serum from B6 $C5^{+/+}$, B6 $C5^{-/-}$ mice, or 40% NHS. Since non-pathogenic *L. biflexa* sorovar Patoc strain Patoc is CS-sensitive and is rapidly killed *in vitro* in the presence of NHS (Barbosa et al., 2009), it was included as a positive control. As a negative control, leptospires were also incubated with heat-inactivated serum from B6 $C5^{+/+}$ mice for 2 h before counting the number of viable cells. The number of viable leptospires in the presence of heat-inactivated serum from B6 $C5^{+/+}$ mice was considered 100%. **Figure 5** shows that while non-pathogenic leptospires *L. biflexa* survives only in C5 deficient mouse serum, LPF is resistant to *in vitro* lysis mediated by MAC formed in the presence of NHS or serum from both B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice strains.

DISCUSSION

One of the first studies of the innate immune response against leptospires pointed to the importance of Toll like receptors (TLR) TLR-2 and TLR-4 to recognize leptospiral pathogen patterns when it was observed that TLR-2 and TLR-4 knockouts rapidly die when infected with this bacterium (Werts et al., 2001). In addition, the role of specific antibodies to control this infection has been studied by several groups (Adler and Faine, 1976; Chassin et al., 2009). (Kobayashi, 2001) observed that guinea pigs, highly susceptible to *Leptospira*, are protected by administering immune serum from convalescent leptospirosis patients. This pointed to the importance of B lymphocytes and antibodies to the acquired immune response in this case.

Since rodents are in general considered resistant to *Leptospira*, we decided to use a murine model to explore the *in vivo* importance of C5 in the first days of infection comparing several parameters in B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice. All animals (male; 4–6 weeks old) survived at least up to 8 days of infection with 1.5×10^8 LPF. Similar results were observed when we infected another C5 deficient (A/J) mouse strain with the same inoculum. Different results were observed by Ratet et al. (2014) when they infected 7–10 weeks old female wild type C57BL/6J mice with 10^8 pathogenic *L. interrogans* serovar Manilae. They reported that all animals developed septicemia which led to death on the third day of infection, indicating that even though mice are considered resistant to leptospires, they can be more or less vulnerable to this infection depending on the combination of *Leptospira* serovar pathogenicity and animal characteristics.

In our model, on the third day of infection we detected a higher LPF number in the liver of infected B6 $C5^{-/-}$ mice when compared to the wild type group, indicating that the CS is important to limit bacterial proliferation during the early days of infection. Since the LPF load in liver is controlled on

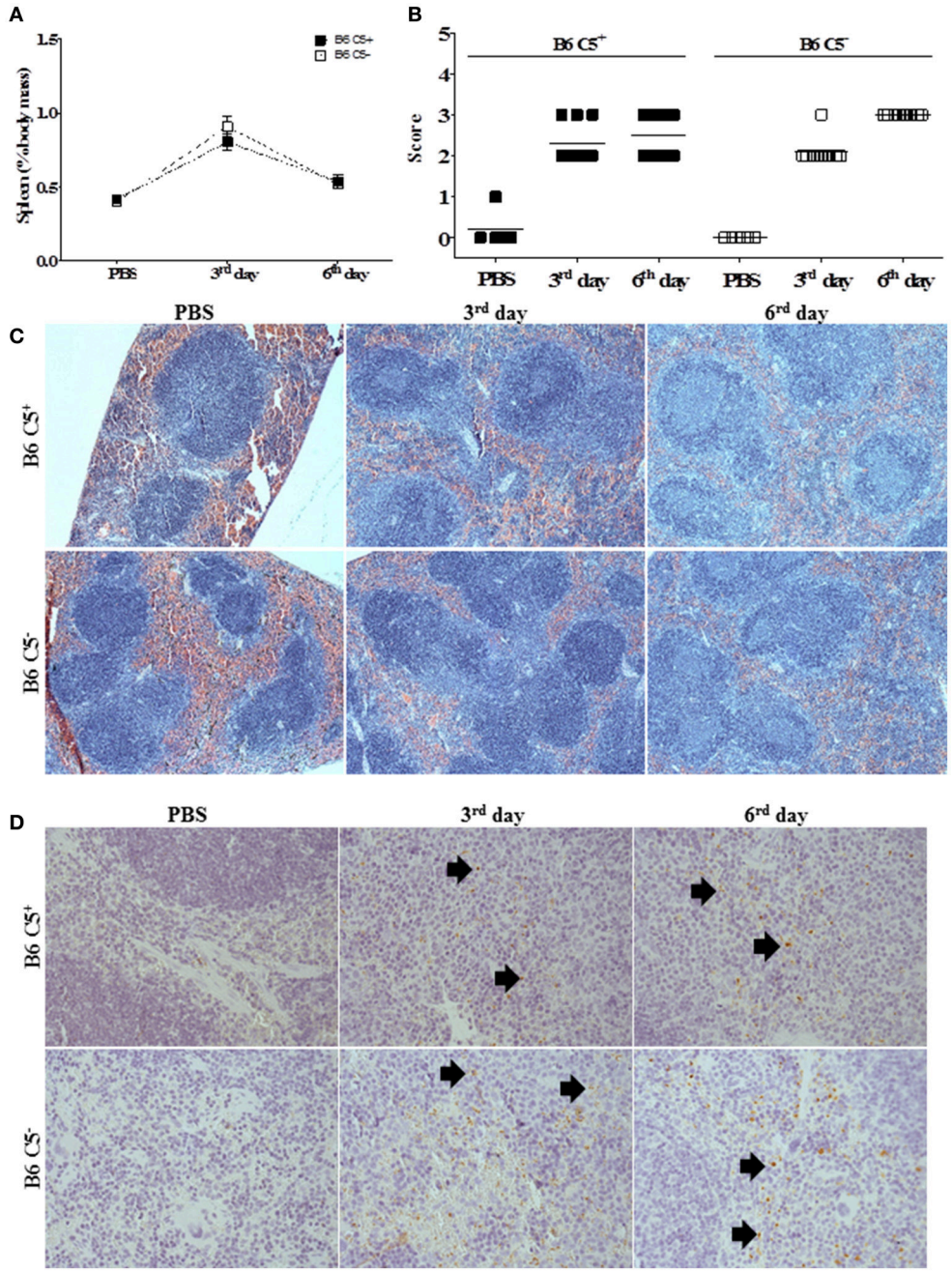


FIGURE 4 | Spleen alterations during LPF infection. Mice were inoculated i/p with 1.5×10^8 LPF or only PBS and then euthanized on the third or sixth day post-infection ($n \geq 5$). **(A)** Splenomegaly was expressed as percentage of spleen mass to the total body mass. **(B)** Score of morphological alterations. **(C)** Spleen sections stained with HE. **(D)** Immunochemical analysis using anti-leptospiral antibodies. Arrows indicate the presence of LPF antigen.

the sixth day in both B6 $C5^{-/-}$ and B6 $C5^{+/+}$ mice, other participants of the innate immune response are acting together. Phagocytic cells such as Kupffer cells could limit the spread of this pathogen in this organ, through Complement receptors (CR) such as CR1, CR3, CR4 (Hinglais et al., 1989), and CRIg (Helmy et al., 2006). It is likely that the release of activated fragments C3b and iC3b, the most important CS opsonins, are generated equally by B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice after activation of the Alternative or Lectin Pathways. This result could also suggest that LPF could be somehow refractory to lysis by MAC. In agreement with this hypothesis, da Silva et al. (2015) demonstrated that LPF is able to bind to human vitronectin, a soluble regulatory protein that binds to C5b67 (Podack et al., 1984; Singh et al., 2010), and consequently

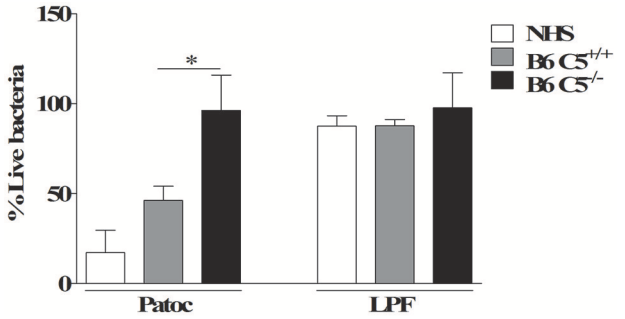


FIGURE 5 | Percentage of viable LPF after *in vitro* incubation with mouse or human serum. 1×10^8 LPF (pathogenic) or non-pathogenic *L. biflexa* sorovar Patoc strain Patoc were incubated for 2 h in 40% of B6 $C5^{+/+}$, B6 $C5^{-/-}$ mice serum or normal human serum (NHS) used here as positive control. The number of viable leptospires was counted using dark-field microscopy. The percentage of viable leptospires in the presence of serum was calculated considering respectively heat-inactivated B6 $C5^{+/+}$ mice serum, heat-inactivated B6 $C5^{-/-}$ mice serum or heat-inactivated NHS considered 100% survival (negative controls). *indicates $p < 0.05$.

inhibits surface MAC formation. It is worth remembering that leptospiral ligands such as LigA, LigB, and LcpA, present exclusively on the surface of pathogenic leptospires, are capable of binding to host Factor H and C4BP to control CS activation on their surface (Castiblanco-Valencia et al., 2012; da Silva et al., 2015). Considering that non-pathogenic leptospires *L. biflexa* survived when incubated *in vitro* with serum from C5 deficient mice, they could possibly survive during infection *in vivo* in these animals. However, this question remains to be investigated, since other components of innate immunity should contribute to the elimination of this spirochete in the host.

Although the survival of mice was independent of the presence of C5, hepatic lesions were observed on the third and sixth days of infection in higher score in the liver of wild type mice, suggesting that the presence of C5 and its fragments leads to a local inflammatory response. Likewise, an increase in the number of Kupffer cells in the sinusoids and leukocyte infiltrates in the liver was observed as previously reported (Chassin et al., 2009; da Silva et al., 2012). In addition, other lesions such as areas of hepatocyte necrosis and destrabecullation were also observed, although at a lower frequency.

Using C3H/HeJ mice, Chen et al. (2017) concluded that macrophages are the main phagocytic cells (predominating over neutrophils) during infection with *L. interrogans* strain Lai. However, neutrophils may also help to control this infection by releasing extracellular traps (NETs) when in contact with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 leading to leptospiral killing (Scharrig et al., 2015). When neutrophils were depleted *in vivo* after use of monoclonal antibody mAb1A8, the number of *L. interrogans* increased in the liver on the third day of infection and in the kidney after 14 days of infection, indicating that neutrophils are important in the early days of infection to control leptospirosis. Neutropenia was observed in other study by Stefos et al. (2005) during murine infections with *L. interrogans*. In contrast, in human leptospirosis, the total number of polymorphonuclear neutrophils increases in the first days post-infection (Raffray et al., 2016).

Increased numbers of circulating monocytes have been observed in cases of sepsis in hospitalized leptospirosis patients (Hoser et al., 2012). Leptospires are able to infect human and murine macrophages (Merien et al., 1997) and once internalized, the bacteria may trigger changes in host cell gene expression, leading to apoptosis (Merien et al., 1997; Hu et al., 2013; Xue et al., 2013). The gene expression alterations observed in macrophages from leptospires-infected organisms might also occur in monocytes present in the peripheral circulation, suggesting that programmed cell death may also be occurring in this cell type (Jin et al., 2009; Xue et al., 2013).

C5 protein is important for the activity of different cell types, including lymphocytes, and its absence is responsible for lower lytic activity of T $CD8^+$ lymphocytes and reduced cytokine synthesis by T $CD4^+$ lymphocytes in different experimental models (Kim et al., 2004; Moulton et al., 2007; Strainic et al., 2008). The interaction of C5a with its receptors present on T $CD4^+$ lymphocytes provides the survival stimuli for these cells *in vitro* (Strainic et al., 2008). Although there are no studies that show the participation of C5 in the viability of T $CD8^+$ lymphocytes, the activation of these cells is facilitated in the presence of C5a (Strainic et al., 2008). In the case of C5 deficient mice, the lack of C5 may have reduced the stimulation that T $CD8^+$ lymphocytes receive to survive and proliferate, resulting in fewer cells in the circulation (Strainic et al., 2008). It has also been shown that the stimulation of T lymphocytes in the presence of C5a reduces the percentage of apoptotic cells and the C5a-C5aR axis stimulates cell expansion (Lalli et al., 2008). The spleen of both C5-deficient and wild-type mouse strains were shown to be a site of much cellular activity during infection. The spleen acts as an active organ in the elimination of circulating leptospires and traces of LPF antigen were found mainly in the red pulp, where macrophages reside.

Finally, it was not possible to observe renal lesions or detect leptospires in the kidneys of the mice in our experimental model through histological and imunochemical analysis. Although the immunohistochemical analysis did not indicate the presence of LPF in the kidneys, the use of more sensitive methods like qPCR would have been better indicated to compare if this leptospiral load would be significantly higher in the absence of C5. Even so, the bacterial load would have been much lower than that observed in the liver. The pathogenesis of acute kidney injury in leptospirosis is the direct nephrotoxic action of the leptospira infection and toxins release, but hemodynamic alteration, jaundice, and rhabdomyolysis (a disruption of skeletal muscle integrity) are also associated (Daher Ede et al., 2010; Abreu et al., 2017). Taking together the biochemical assay results regarding the elevation of AST only in $C5^{-/-}$ mice on the third day post infection and uric acid in both mice on the sixth day post infection, we can suggest that the infection may be inducing rhabdomyolysis in a manner independent of C5. Although the kidney is an important organ for infection because

it is a site where leptospires are fixed and later eliminated with the urine, apparently LPF was not able to colonize this organ in the first week of infection. It is possible that the serovar used in our laboratory requires longer periods of time to reach the kidney. Chassin et al. (2009) also observed relatively low leptospiral load on the third day of infection in the kidney and lung from wild type C57Bl/6J mice infected with pathogenic *L. interrogans* serovar Copenhageni strain Fiocruz LI-130.

Our results suggest that C5 may play a role in the direct killing of LPF only up to the third day of infection *in vivo*. Considering the inflammatory properties of the C5a fragment, the presence of C5 is associated with tissue lesions observed in the liver of mice infected with *L. interrogans*. However, this variation did not significantly alter the ability of mice to control infection caused by LPF, suggesting a minor role for C5 in this infection model. The possibility that C3b/iC3b opsonins play a more important role than the MAC ($C5b\text{-}9_n$) or the fragment C5a to control leptospiral murine infection remains to be further investigated.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethics Committee on Animal Experimentation of Institute of Biomedical Sciences from the University of São Paulo. The protocol was approved according to the Certificate 061/10/CEEA.

AUTHOR CONTRIBUTIONS

IdC and LB did the experiments; TF, LB, and AG-M helped with the leptospires cultures and counting viable leptospires; MA determined the serum concentrations of cytokines; AdS helped with immunochemistry analyses, SV provided the cultures and confirmed the pathogenicity of leptospires, IdC, LB, and LI designed the experiments, analyzed the results and wrote the manuscript.

FUNDING

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Proc.# 2015/09972-0 and # 2010/50043-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Proc#309907/2013-6).

ACKNOWLEDGMENTS

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). IdC and LB were recipients of graduate fellowships from FAPESP. The authors would like to thank Dr. Thales de Brito for histopathological analysis, Paulo Albe for histology technical support, Rosana Prisco for statistical analysis and all biochemical kits kindly provided by Bioclin Quibasa. The authors thank Dr. Shaker Chuck Farah, Dr. Angela Silva Barbosa, and Elena Lazara Santiesteban Lores for critically reviewing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00063/full#supplementary-material

Supplementary Figure 1 | Immunohistochemical analysis of liver from infected mice. Mice were inoculated i/p with 1.5×10^8 LPF or PBS and then euthanized on the third or the sixth day, when the liver was collected for LPF antigen analysis ($n \geq 5$). The arrows indicate the presence of labeled LPF antigens.

Supplementary Figure 2 | Serum concentrations of hepatic ALT **(A)** and AST **(B)** enzymes in infected mice. Mice were inoculated i/p with 1.5×10^8 LPF or PBS and then euthanized on the third or the sixth day, when the blood was collected for analysis ($n \geq 5$). ALT and AST were used as indicators of liver damage. The significant differences ($p < 0.05$) are represented as follows: [b]vs. third day and [c]vs. B6 $C5^{+/+}$.

Supplementary Figure 3 | Histopathological analysis of lung from infected mice. Mice were inoculated i/p with 1.5×10^8 LPF or PBS and then euthanized on the third or the sixth day, when the lung was collected for histopathological analysis ($n \geq 5$). **(A)** The liver sections (3–5 micrometers) were stained with HE and evaluated at 200x magnification. **(B)** Scores of histopathological alterations.

Supplementary Figure 4 | Histopathological analysis of kidney from LPF infected mice. Mice were inoculated i/p with 1.5×10^8 LPF or PBS and then euthanized on the third or the sixth days, when the kidney was collected for histopathological analysis ($n \geq 5$). **(A)** The kidney sections (3–5 micrometers) were stained with HE, 200x magnification. (**B**) Serum levels of urea and uric acid. The significant differences ($p < 0.05$) are represented as follows: [a]vs. PBS; [b]vs. third day.

Supplementary Figure 5 | Concentrations of pro- and anti-inflammatory cytokines during infection by LPF. B6 $C5^{+/+}$ and B6 $C5^{-/-}$ mice were inoculated i/p with 1.5×10^8 LPF or PBS and then euthanized on the third or the sixth day, when the liver was collected and prepared for cytokine concentration determination ($n \geq 5$). Liver cells were disrupted and treated with protease inhibitors. The concentration of several cytokines **(A–F)** was analyzed in the liver extracts by ELISA (normalized with respect to total protein) or in the serum of infected mice ($n \geq 3$) by CBA **(G–I)**. un: undetected. The significant differences ($p < 0.05$) are represented as follows: [a]vs. PBS; [c]B6 $C5^{+/+}$vs. B6 $C5^{-/-}$.

REFERENCES

Abreu, P. A. E., Seguro, A. C., Canale, D., Silva, A. M. G. D., and Matos, L. D. R. B. (2017). Lp25 membrane protein from pathogenic *Leptospira* spp. is associated with rhabdomyolysis and oliguric acute kidney injury in a guinea pig model of leptospirosis. *PLOS Negl. Trop. Dis.* 11:e0005615. doi: 10.1371/journal.pntd.0005615

Adler, B. (2015). History of leptospirosis and leptospira. *Curr. Top. Microbiol. Immunol.* 387, 1–9. doi: 10.1007/978-3-662-45059-8_1

Adler, B., and Faine, S. (1976). Susceptibility of mice treated with cyclophosphamide to lethal infection with *Leptospira interrogans* Serovar pomona. *Infect. Immun.* 14, 703–708.

Aguilar-Ramirez, P., Reis, E. S., Florido, M. P., Barbosa, A. S., Farah, C. S., Costa-Carvalho, B. T., et al. (2009). Skipping of exon 30 in C5 gene results in complete human C5 deficiency and demonstrates the importance of C5d and CUB domains for stability. *Mol. Immunol.* 46, 2116–2123. doi: 10.1016/j.molimm.2008.10.035

Amamura, T. A., Fraga, T. R., Vasconcellos, S. A., Barbosa, A. S., and Isaac, L. (2017). Pathogenic Leptospira secreted proteases target the membrane attack complex: a potential role for thermolysin in complement inhibition. *Front. Microbiol.* 8:958. doi: 10.3389/fmicb.2017.00958

Barbosa, A. S., Abreu, P. A., Vasconcellos, S. A., Morais, Z. M., Gonçales, A. P., Silva, A. S., et al. (2009). Immune evasion of leptospira species

by acquisition of human complement regulator C4BP. *Infect. Immun.* 77, 1137–1143. doi: 10.1128/IAI.01310-08

Barbosa, A. S., Monaris, D., Silva, L. B., Morais, Z. M., Vasconcellos, S. A., Cianciarullo, A. M., et al. (2010). Functional characterization of LcpA, a surface-exposed protein of Leptospira spp. that binds the human complement regulator C4BP. *Infect. Immun.* 78, 3207–3216. doi: 10.1128/IAI.00279-10

Bavia, L., de Castro, Í. A., and Isaac, L. (2015). C57BL/6 and A/J mice have different inflammatory response and liver lipid profile in experimental alcoholic liver disease. *Mediat. Inflamm.* 2015:491641. doi: 10.1155/2015/491641

Bavia, L., de Castro, Í. A., Massironi, S. M., and Isaac, L. (2014). Basal physiological parameters of two congenic mice strains: C5 deficient C57BL/6 and C5 sufficient A/J. *Immunol. Lett.* 159, 47–54. doi: 10.1016/j.imlet.2014.02.010

Breda, L. C., Hsieh, C. L., Castiblanco-Valencia, M. M., da Silva, L. B., Barbosa, A. S., Blom, A. M., et al. (2015). Fine mapping of the interaction between C4b-binding protein and outer membrane proteins LigA and LigB of pathogenic *Leptospira interrogans*. *PLoS Negl. Trop. Dis.* 9:e0004192. doi: 10.1371/journal.pntd.0004192

Castiblanco-Valencia, M. M., Fraga, T. R., Breda, L. C., Vasconcellos, S. A., Figueira, C. P., Picardeau, M., et al. (2016). Acquisition of negative complement regulators by the saprophyte Leptospira biflexa expressing LigA or LigB confers enhanced survival in human serum. *Immunol. Lett.* 173, 61–68. doi: 10.1016/j.imlet.2016.03.005

Castiblanco-Valencia, M. M., Fraga, T. R., Silva, L. B., Monaris, D., Abreu, P. A., Strobel, S., et al. (2012). Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *J. Infect. Dis.* 205, 995–1004. doi: 10.1093/infdis/jir875

Chassin, C., Picardeau, M., Goujon, J. M., Bourhy, P., Quellard, N., Darche, S., et al. (2009). TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial pathogen, *Leptospira interrogans*. *J. Immunol.* 183, 2669–2677. doi: 10.4049/jimmunol.0900506

Chen, X., Li, S. J., Ojcius, D. M., Sun, A. H., Hu, W. L., Lin, X., et al. (2017). Mononuclear-macrophages but not neutrophils act as major infiltrating anti-leptospiral phagocytes during leptospirosis. *PLoS ONE* 12:e0181014. doi: 10.1371/journal.pone.0181014

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

Daher Ede, F., de Abreu, K. L., and da Silva Junior, G. B. (2010). Leptospirosis-associated acute kidney injury. *J. Bras. Nefrol.* 32, 400–407. doi: 10.1590/S0101-28002010000400010

da Silva, J. B., Carvalho, E., Covarrubias, A. E., Ching, A. T., Mattaraia, V. G., Paiva, D., et al. (2012). Induction of TNF-alfa and CXCL-2 mRNAs in different organs of mice infected with pathogenic Leptospira. *Microb. Pathog.* 52, 206–216. doi: 10.1016/j.micpath.2012.01.002

da Silva, L. B., Miragaia Ldos, S., Breda, L. C., Abe, C. M., Schmidt, M. C., Moro, A. M., et al. (2015). Pathogenic Leptospira species acquire factor H and vitronectin via the surface protein LcpA. *Infect. Immun.* 83, 888–897. doi: 10.1128/IAI.02844-14

Ferrer, M. F., Scharrig, E., Alberdi, L., Cedola, M., Pretre, G., Drut, R., et al. (2014). Decay-accelerating factor 1 deficiency exacerbates leptospiral-induced murine chronic nephritis and renal fibrosis. *PLoS ONE* 9:e102860. doi: 10.1371/journal.pone.0102860

Fraga, T. R., Courrol, Ddos, S., Castiblanco-Valencia, M. M., Hirata, I. Y., Vasconcellos, S. A., Juliano, L., et al. (2014). Immune evasion by pathogenic Leptospira strains: the secretion of proteases that directly cleave complement proteins. *J. Infect. Dis.* 209, 876–886. doi: 10.1093/infdis/jit569

Fraga, T. R., Isaac, L., and Barbosa, A. S. (2016). Complement evasion by Pathogenic Leptospira. *Front. Immunol.* 7:623. doi: 10.3389/fimmu.2016.00623

Gomes-Solecki, M., Santecchia, I., and Werts, C. (2017). Animal models of Leptospirosis: of mice and hamsters. *Front. Immunol.* 8:58. doi: 10.3389/fimmu.2017.00058

Guo, R. F., and Ward, P. A. (2005). Role of C5a in inflammatory responses. *Annu. Rev. Immunol.* 23, 821–852. doi: 10.1146/annurev.immunol.23.021704.115835

Helmy, K. Y., Katschke, K. J., Gorgani, N. N., Kljavin, N. M., Elliott, J. M., Diehl, L., et al. (2006). CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* 124, 915–927. doi: 10.1016/j.cell.2005.12.039

Hinglais, N., Kazatchkine, M. D., Mandet, C., Appay, M. D., and Bariety, J. (1989). Human liver Kupffer cells express CR1, CR3, and CR4 complement receptor antigens. An immunohistochemical study. *Lab. Invest.* 61, 509–514.

Hoser, G. A., Skirecki, T., Złotorowicz, M., Zielinska-Borkowska, U., and Kawiak, J. (2012). Absolute counts of peripheral blood leukocyte subpopulations in intraabdominal sepsis and pneumonia-derived sepsis: a pilot study. *Folia Histochem. Cytobiol.* 50, 420–426. doi: 10.5603/FHC.2012.0057

Hu, W., Ge, Y., Ojcius, D. M., Sun, D., Dong, H., Yang, X. F., et al. (2013). p53 signalling controls cell cycle arrest and caspase-independent apoptosis in macrophages infected with pathogenic Leptospira species. *Cell. Microbiol.* 15, 1642–1659. doi: 10.1111/cmi.12141

Jin, D., Ojcius, D. M., Sun, D., Dong, H., Luo, Y., Mao, Y., et al. (2009). *Leptospira interrogans* induces apoptosis in macrophages via caspase-8- and caspase-3-dependent pathways. *Infect. Immun.* 77, 799–809. doi: 10.1128/IAI.00914-08

Kim, A. H., Dimitriou, I. D., Holland, M. C., Mastellos, D., Mueller, Y. M., Altman, J. D., et al. (2004). Complement C5a receptor is essential for the optimal generation of antiviral CD8+ T cell responses. *J. Immunol.* 173, 2524–2529. doi: 10.4049/jimmunol.173.4.2524

Ko, A. I., Goarant, C., and Picardeau, M. (2009). Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* 7, 736–747. doi: 10.1038/nrmicro2208

Kobayashi, Y. (2001). Clinical observation and treatment of leptospirosis. *J. Infect. Chemother.* 7, 59–68. doi: 10.1007/s101560100011

Kotimaa, J., Klar-Mohammad, N., Gueler, F., Schilders, G., Jansen, A., Rutjes, H., et al. (2016). Sex matters: systemic complement activity of female C57BL/6J and BALB/cJ mice is limited by serum terminal pathway components. *Mol. Immunol.* 76, 13–21. doi: 10.1016/j.molimm.2016.06.004

Lalli, P. N., Strainic, M. G., Yang, M., Lin, F., Medof, M. E., and Heeger, P. S. (2008). Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. *Blood* 112, 1759–1766. doi: 10.1182/blood-2008-04-151068

Macedo, A. C., and Isaac, L. (2016). Systemic lupus erythematosus and deficiencies of early components of the complement classical pathway. *Front. Immunol.* 7:55. doi: 10.3389/fimmu.2016.00055

Markiewski, M. M., DeAngelis, R. A., Strey, C. W., Foukas, P. G., Gerard, C., Gerard, N., et al. (2009). The regulation of liver cell survival by complement. *J. Immunol.* 182, 5412–5418. doi: 10.4049/jimmunol.0804179

Markiewski, M. M., Mastellos, D., Tudoran, R., De Angelis, R. A., Strey, C. W., Franchini, S., et al. (2004). C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. *J. Immunol.* 173, 747–754. doi: 10.4049/jimmunol.173.2.747

Meri, T., Murgia, R., Stefanel, P., Meri, S., and Cinco, M. (2005). Regulation of complement activation at the C3-level by serum resistant leptospires. *Microb. Pathog.* 39, 139–147. doi: 10.1016/j.micpath.2005.07.003

Merien, F., Baranton, G., and Perolat, P. (1997). Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. *Infect. Immun.* 65, 729–738.

Moulton, R. A., Mashruwala, M. A., Smith, A. K., Lindsey, D. R., Wetsel, R. A., Haviland, D. L., et al. (2007). Complement C5a anaphylatoxin is an innate determinant of dendritic cell-induced Th1 immunity to Mycobacterium bovis BCG infection in mice. *J. Leukoc. Biol.* 82, 956–967. doi: 10.1189/jlb.0206119

Nicholson-Weller, A., Burge, J., and Austen, K. F. (1981). Purification from guinea pig erythrocyte stroma of a decay-accelerating factor for the classical c3 convertase, C4b,2a. *J. Immunol.* 127, 2035–2039.

Nicholson-Weller, A., March, J. P., Rosenfeld, S. I., and Austen, K. F. (1983). Affected erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are deficient in the complement regulatory protein, decay accelerating factor. *Proc. Natl. Acad. Sci. U.S.A.* 80, 5066–5070. doi: 10.1073/pnas.80.16.5066

Podack, E. R., Preissner, K. T., and Müller-Eberhard, H. J. (1984). Inhibition of C9 polymerization within the SC5b-9 complex of complement by S-protein. *Acta Pathol. Microbiol. Immunol. Scand. Suppl.* 284, 89–96.

Raffray, L., Giry, C., Vandroux, D., Kuli, B., Randrianjohany, A., Pequin, A. M., et al. (2016). Major neutrophilia observed in acute phase of human leptospirosis is not associated with increased expression of granulocyte cell activation markers. *PLoS ONE* 11:e0165716. doi: 10.1371/journal.pone.0165716

Ratet, G., Veyrier, F. J., Fanton d'Andon, M., Kammerscheit, X., Nicola, M. A., Picardeau, M., et al. (2014). Live imaging of bioluminescent *Leptospira interrogans* in mice reveals renal colonization as a stealth escape from the blood defenses and antibiotics. *PLoS Negl. Trop. Dis.* 8:e3359. doi: 10.1371/journal.pntd.0003359

Scharrig, E., Carestia, A., Ferrer, M. F., Cédola, M., Pretre, G., Drut, R., et al. (2015). Neutrophil extracellular traps are involved in the innate immune response to infection with leptospira. *PLoS Negl. Trop. Dis.* 9:e0003927. doi: 10.1371/journal.pntd.0003927

Serna, M., Giles, J. L., Morgan, B. P., and Bubeck, D. (2016). Structural basis of complement membrane attack complex formation. *Nat. Commun.* 7:10587. doi: 10.1038/ncomms10587

Singh, B., Su, Y. C., and Riesbeck, K. (2010). Vitronectin in bacterial pathogenesis: a host protein used in complement escape and cellular invasion. *Mol. Microbiol.* 78, 545–560. doi: 10.1111/j.1365-2958.2010.07373.x

Stefos, A., Georgiadou, S. P., Gioti, C., Loukopoulos, A., Ioannou, M., Pournaras, S., et al. (2005). Leptospirosis and pancytopenia: two case reports and review of the literature. *J. Infect.* 51, e277–e280. doi: 10.1016/j.jinf.2005.03.008

Strainic, M. G., Liu, J., Huang, D., An, F., Lalli, P. N., Muqim, N., et al. (2008). Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 28, 425–435. doi: 10.1016/j.immuni.2008.02.001

Strey, C. W., Markiewski, M., Mastellos, D., Tudoran, R., Spruce, L. A., Greenbaum, L. E., et al. (2003). The proinflammatory mediators C3a and C5a are essential for liver regeneration. *J. Exp. Med.* 198, 913–923. doi: 10.1084/jem.20030374

Torgerson, P. R., Hagan, J. E., Costa, F., Calcagno, J., Kane, M., Martinez-Silveira, M. S., et al. (2015). Global burden of leptospirosis: estimated in terms of disability adjusted life years. *PLoS Negl. Trop. Dis.* 9:e0004122. doi: 10.1371/journal.pntd.0004122

Vieira, M. L., Atzingen, M. V., Oliveira, T. R., Oliveira, R., Andrade, D. M., Vasconcellos, S. A., et al. (2010). *In vitro* identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS ONE* 5:e11259. doi: 10.1371/journal.pone.0011259

Vieira, M. L., de Morais, Z. M., Vasconcellos, S. A., Romero, E. C., and Nascimento, A. L. (2011). *In vitro* evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. *Microb. Pathog.* 51, 360–365. doi: 10.1016/j.micpath.2011.06.008

Ward, P. A. (2010). The harmful role of C5a on innate immunity in sepsis. *J. Innate Immun.* 2, 439–445. doi: 10.1159/000317194

Werts, C., Tapping, R. I., Mathison, J. C., Chuang, T. H., Kravchenko, V., Saint Girons, I., et al. (2001). Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat. Immunol.* 2, 346–352. doi: 10.1038/86354

Xue, F., Zhao, X., Yang, Y., Zhao, J., Yang, Y. Cao, Y., et al. (2013). Responses of murine and human macrophages to leptospiral infection: a study using comparative array analysis. *PLoS Negl. Trop. Dis.* 7:e2477. doi: 10.1371/journal.pntd.0002477

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 09 August 2017
doi: 10.3389/fcimb.2017.00362

Pathogenic Leptospires Modulate Protein Expression and Post-translational Modifications in Response to Mammalian Host Signals

Jarlath E. Nally[1]*, *Andre A. Grassmann*[2,3], *Sébastien Planchon*[4], *Kjell Sergeant*[4], *Jenny Renaut*[4], *Janakiram Seshu*[5], *Alan J. McBride*[2,6] and *Melissa J. Caimano*[3]

[1] *Infectious Bacterial Diseases Research, National Animal Disease Center, United States Department of Agriculture, Agricultural Research Service, Ames, IA, United States,* [2] *Biotechnology Unit, Technological Development Center, Federal University of Pelotas, Pelotas, Brazil,* [3] *Departments of Medicine, Pediatrics, and Molecular Biology and Biophysics, University of Connecticut Health Center, Farmington, CT, United States,* [4] *Environmental Research and Innovation Department, Luxembourg Institute of Science and Technology, Belvaux, Luxembourg,* [5] *Department of Biology, University of Texas San Antonia, San Antonia, TX, United States,* [6] *Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Ministry of Health, Salvador, Brazil*

Pathogenic species of *Leptospira* cause leptospirosis, a bacterial zoonotic disease with a global distribution affecting over one million people annually. Reservoir hosts of leptospirosis, including rodents, dogs, and cattle, exhibit little to no signs of disease but shed large numbers of organisms in their urine. Transmission occurs when mucosal surfaces or abraded skin come into contact with infected urine or urine-contaminated water or soil. Whilst little is known about how *Leptospira* adapt to and persist within a reservoir host, *in vitro* studies suggest that leptospires alter their transcriptomic and proteomic profiles in response to environmental signals encountered during mammalian infection. We applied the dialysis membrane chamber (DMC) peritoneal implant model to compare the whole cell proteome of *in vivo* derived leptospires with that of leptospires cultivated *in vitro* at 30°C and 37°C by 2-dimensional difference in-gel electrophoresis (2-D DIGE). Of 1,735 protein spots aligned across 9 2-D DIGE gels, 202 protein spots were differentially expressed ($p < 0.05$, fold change >1.25 or < -1.25) across all three conditions. Differentially expressed proteins were excised for identification by mass spectrometry. Data are available via ProteomeXchange with identifier PXD006995. The greatest differences were detected when DMC-cultivated leptospires were compared with IV30- or IV37-cultivated leptospires, including the increased expression of multiple isoforms of Loa22, a known virulence factor. Unexpectedly, 20 protein isoforms of LipL32 and 7 isoforms of LipL41 were uniformly identified by DIGE as differentially expressed, suggesting that unique post-translational modifications (PTMs) are operative in response to mammalian host conditions. To test this hypothesis, a rat model of persistent renal colonization was used to isolate leptospires directly from the urine of experimentally infected rats. Comparison of urinary derived leptospires to IV30 leptospires by 2-D immunoblotting confirmed that modification of proteins with trimethyllysine and acetyllysine occurs to a different degree in response to mammalian host signals

OPEN ACCESS

Edited by:
Azad Eshghi,
University of Victoria Genome British Columbia Proteomics Centre, Canada

Reviewed by:
Philip E. Stewart,
National Institute of Allergy and Infectious Diseases (NIH), United States
Caroline Cameron,
University of Victoria, Canada

***Correspondence:**
Jarlath E. Nally
Jarlath.nally@ars.usda.gov

Received: *31 May 2017*
Accepted: *26 July 2017*
Published: *09 August 2017*

Citation:
Nally JE, Grassmann AA, Planchon S, Sergeant K, Renaut J, Seshu J, McBride AJ and Caimano MJ (2017) Pathogenic Leptospires Modulate Protein Expression and Post-translational Modifications in Response to Mammalian Host Signals. Front. Cell. Infect. Microbiol. 7:362. doi: 10.3389/fcimb.2017.00362

encountered during persistent renal colonization. These results provide novel insights into differential protein and PTMs present in response to mammalian host signals which can be used to further define the unique equilibrium that exists between pathogenic leptospires and their reservoir host of infection.

Keywords: *Leptospira*, spirochetes, proteomics, DIGE, post-translational modifications

INTRODUCTION

Pathogenic species of *Leptospira* cause leptospirosis, a bacterial zoonotic disease with a global distribution affecting over one million people annually (Costa et al., 2015). Leptospires colonize renal tubules and are excreted in urine from reservoir hosts of infection. Contact with contaminated urine or water sources can result in infection via breaches of the skin and/or mucosal surfaces and disseminate haematogenously, causing a range of clinical symptoms from mild fever, to icteric Weil's disease and pulmonary hemorrhage syndrome. Mortality rates range from 10 to 70% depending on disease severity (McBride et al., 2005). In developed countries, leptospirosis is primarily a recreational disease, an occupational disease of farm workers, veterinarians, and slaughter plant workers, and in returning travelers. In developing countries, it is a socioeconomic disease perpetuated by rapid urbanization, rodent infestation, and transmission via contaminated water sources associated with limited infrastructures and severe weather events. Both rodents and domestic farm animal species serve as reservoir hosts of infection and sources of disease transmission to humans.

A large body of work has demonstrated that leptospires regulate and modify gene expression in response to environmental cues, as encountered during disease transmission, including changes in temperature (Lo et al., 2006; Qin et al., 2006; Matsunaga et al., 2013), osmolarity (Matsunaga et al., 2007), concentration of iron (Lo et al., 2010), the presence of serum (Patarakul et al., 2010), and interaction with macrophages (Xue et al., 2010). However, since leptospires are not readily amenable to genetic manipulation, the functional and biological significance of these transcriptomic changes are unclear (Adler et al., 2011; Picardeau, 2015). There often is limited correlation between gene and protein expression by leptospires (Lo et al., 2009) but it is clear that the protein profiles of pathogenic leptospires also are regulated in response to environmental cues encountered during host infection (e.g., in response to temperature and iron depletion; Nally et al., 2001a,b; Cullen et al., 2002; Eshghi et al., 2009). More recently, it was shown that protein expression, in response to changing environmental conditions, can be modified further by specific post-translational modifications (PTM; Eshghi et al., 2012). Indeed, both saprophytic and pathogenic leptospires have comprehensive bio-systems to modify proteins (Cao et al., 2010; Schmidt et al., 2011; Stewart et al., 2016). Recently, 32 phosphorylated, 46 acetylated, and 155 methylated proteins were identified that not only confirmed multiple modifications in prokaryotes, but also suggests that *L. interrogans* shares significant similarities with protein modification systems in eukaryotes (Cao et al., 2010). The surface-exposed outer membrane protein, OmpL32, undergoes differential methylation of glutamic acid residues in response to modifying *in vitro* growth conditions that emulate those encountered during mammalian host infection (Eshghi et al., 2012). In addition, *L. interrogans* can utilize endogenous biosynthetic pathways to elaborate surface structures containing sialic acids and related nonulosonic acids (Ricaldi et al., 2012b). Finally, the detection of PTM on lysine residues within LipL32 from *in vivo*-isolated *L. interrogans* implies that the infection-generated modification of leptospiral proteins may have a biologically relevant function during the course of infection (Witchell et al., 2014).

The paucibacillary nature of spirochetal infections, combined with the challenges associated with acquiring pathogens free from contaminating host proteins, makes the study of these bacteria in a mammalian host-adapted state inherently difficult. As an alternative approach, we developed a novel animal model in which leptospires are cultivated in a dialysis membrane chambers (DMCs) implanted within the peritoneal cavity of rats, where they are exposed to some of the environmental cues encountered during host infection (Caimano et al., 2014). This strategy has been applied successfully to compare the transcriptome of *L. interrogans* cultivated within DMCs with that of leptospires grown under standard *in vitro* conditions. In addition to determining the relative expression levels of "core" housekeeping genes under both growth conditions, we identified 166 genes that were differentially-expressed at the mRNA level by *L. interrogans* in response to mammalian host signals (Caimano et al., 2014).

In the current study, we applied the DMC model to compare the proteome of *in vivo*-derived leptospires with that of leptospires cultivated *in vitro* at 30°C or 37°C by 2-dimensional difference in-gel electrophoresis (2-D DIGE). Our analysis indicates that the abundance of leptospiral proteins is modulated in response to mammalian host signals, and not temperature alone. In addition, we confirm that in several proteins there is a change in the presence of the PTM trimethyllysine and acetyllysine in response to environmental cues encountered during persistent renal colonization in a reservoir host of infection. These results provide novel insights into the proteome, including PTM, in response to mammalian host signals, which can be used to further define the unique equilibrium that exists between pathogenic leptospires and their reservoir host of infection.

MATERIALS AND METHODS

In vitro-Cultivated Bacteria

Virulent low-passage *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, kindly provided by

Dr. David Haake (UCLA), was cultivated *in vitro* under standard static conditions for 8–9 days at 30 or 37°C in EMJH medium supplemented with 1% rabbit serum (Pel-Freez Biologicals, Rogers, AR) with 100 μg/ml 5-fluorouracil. Cultures were grown to late logarithmic phase (1–3 × 10^8 per ml). Virulent low-passage *L. interrogans* serogroup Icterohaemorrhagiae strain RJ19115 was cultivated under standard static conditions at 30°C in EMJH medium until late logarithmic phase (1–3 × 10^8 per ml).

Dialysis Membrane Chamber (DMC)-Cultivated Bacteria

All animal experimentation was conducted in accordance with protocols as reviewed and approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee. To obtain mammalian host-adapted organisms, virulent low-passage *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was cultivated in DMCs as previously described (Caimano et al., 2014; Grassmann et al., 2015). In brief, DMCs were prepared with 8–10 ml of EMJH medium [supplemented with 10% vaccine-grade bovine serum albumin (EMD Millipore, Billerica, MA) to maintain osmotic pressure] at a starting inoculum of 10^4 organisms per ml. Using strict aseptic technique, each DMC was implanted into the peritoneal cavity of an anesthetized female Sprague-Dawley rat (Harlan). Approximately 9–10 days later, DMCs were explanted and the quality of leptospires evaluated for motility and density (1–3 × 10^8 leptospires per ml) by dark field microscopy using a Petroff-Hausser counting chamber (Hausser Scientific Co., Horsham, PA).

Urinary Derived Leptospires

All animal experimentation was conducted in accordance with protocols as reviewed and approved by the Animal Care & Use Committee at the National Animal Disease Center, and as approved by USDA Institutional guidelines. Urinary derived leptospires were collected from experimentally infected rats as previously described with slight modification (Monahan et al., 2008; Bonilla-Santiago and Nally, 2011). Male Sprague-Dawley rats (Harlan) of ∼5 weeks of age were experimentally infected with 1 × 10^7 *L. interrogans* strain RJ19115 by intraperitoneal injection, a stain of *L. interrogans* serogroup Icterohaemorrhagiae which results in significant numbers of leptospires excreted in urine from colonized renal tubules. At 2 weeks post-infection (Rojas et al., 2010), rats were housed overnight in metabolism cages and urine collected directly into a 50 ml conical tube containing 0.5 ml Urine preservative (Norgen Biotek Corp). Urine samples were centrifuged at 4°C, 1,000 × g, for 5 min to remove excess debris. Supernatants were then centrifuged at 6,100 × g, 4°C for 60 min to collect urinary derived leptospires. Pellets were resuspended in 1 ml ice-cold 10 mM Tris-Cl, 1 mM EDTA, transferred to a microfuge tube and collected by centrifugation at 12,000 × g, 4°C for 30 min. Samples were frozen at −20°C until analysis.

2-Dimensional Fluorescence Difference In-Gel Electrophoresis (2-D DIGE)

2-D DIGE was performed on 18 biological replicates of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 comprising 6 biological replicates of leptospires cultivated at 30°C, 6 biological replicates of leptospires cultivated at 37°C and 6 biological replicates of leptospires cultivated in DMCs. *In vitro*- and DMC-cultivated leptospires were harvested at late logarithmic phase by centrifugation, washed twice with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.4) and resuspended in solubilization buffer (7 M Urea, 2 M Thiourea and 1% ASB-14) as previously described (Caimano et al., 2014). Protein concentrations were determined using the DC protein assay kit (Bio-Rad) as per manufacturer's instructions. An internal standard was prepared using a mixture of equal amounts of all 18 replicate samples included in the analysis. This internal standard was included in all gels to allow normalization of each independent gel and cross-gel comparison of each spot's density. 2-D DIGE was performed as previously described (Schuller et al., 2015) using 24 cm IPG strips pH 3–7 NL. Labeled samples were mixed according to the experimental design (**Table 1**) and the volume adjusted to 150 μl with rehydration buffer comprising 7 M Urea, 2 M Thiourea, 0.5% (w/v) CHAPS and 2% (v/v) ampholytes. After an overnight rehydration of the strips in 450 μl of Destreak Rehydration Solution (GE Healthcare) complemented with 2% ampholytes, the samples were cup-loaded and focused to reach a total of 75 kVh (150 V for 3 h, 300 V for 3 h, a gradient to 1,000 V over 6 h, a gradient to 10,000 V over 3 h, and 10,000 V for 6.25 h). The second dimension was carried out on an HPE system with large 12% non-fluorescent gels (Serva) following manufacturer's instructions.

Image Acquisition and Analysis

Gels were scanned using the Typhoon® FLA 9500 (GE Healthcare, Buckinghamshire, UK) as per manufacturer's instructions. Gels were analyzed with the Decyder version 7.0. Spot volumes were compared between samples derived from leptospires cultivated at 30°C, 37°C or in DMCs. For each spot, the ratio between conditions is calculated; when the ratio is above one, the fold change equals the ratio and if the ratio is

TABLE 1 | 2-D DIGE experimental design.

Gel #	Cy3	Cy5	Cy2
1	IV37-Sample 2	DMC-Sample 1	Internal standard
2	IV30-Sample 9	IV37-Sample 7	Internal standard
3	DMC-Sample 2	IV30-Sample 3	Internal standard
4	IV30-Sample 1	DMC-Sample 5	Internal standard
5	IV37-Sample 1	IV30-Sample 8	Internal standard
6	DMC-Sample 3	IV37-Sample 9	Internal standard
7	IV37-Sample 8	DMC-Sample 7	Internal standard
8	IV30-Sample 2	IV37-Sample 3	Internal standard
9	DMC-Sample 6	IV30-Sample 7	Internal standard

Leptospires were cultured under in vitro conditions at 30°C (IV30) or 37°C (IV37) and compared with leptospires cultured in dialysis membrane chambers (DMC) as described.

below 1, the fold change is given as −(1/ratio). Statistically significantly expressed protein spots were defined as having a *t*-test $p < 0.05$ and a fold change of >1.25 or < −1.25 (**Supplementary Table 1**). Spots not meeting these statistical criteria were considered non-differentially expressed.

Protein Digestion and Identification

Spots of interest were picked using an Ettan spot picker (GE Healthcare) and digested with a fully automated Evo 2 workstation (Tecan). In brief, excised spots are washed and the proteins reduced with DTT and alkylated with iodoacetamide. After removal of excess salts, proteins were digested for 6 h at 37°C with 40 ng of trypsin in 50 mM ammonium carbonate (TrypsinGold, Promega). After digestion, peptides were extracted from the gel pieces, the extracts dried and spotted onto a MALDI plate. Mass spectra were acquired using a MALDI-TOF-TOF mass spectrometer (Sciex 5800) and after the acquisition of a MS spectrum, the 10 highest peaks (excluding known contaminants) are selected for fragmentation. The MS-spectrum and the 10 MS/MS spectra are grouped in the database search with an in-house MASCOT engine (version 2.3, Matrix Science, matrixscience.com, London, UK). The primary database searched was the "*L. interrogans* serovar Copenhageni str. Fiocruz L1-130" database, downloaded on the 6th of February 2014 from NCBI (txid267671) and containing 7,818 sequences. When high-quality spectra were not matched during this primary search, they were individually resubmitted to a search against the entire NCBInr database (downloaded on the 23rd of September 2013 and containing 32,770,904 sequences). This resulted in the identification of bovine serum albumin (BSA) in some of the spots (**Supplementary Table 2**), a common contaminant due to the high concentration of BSA in the EMJH used for DMCs. The following parameters were used for the database searches: carbamidomethyl (C) as fixed modification; dioxidation (W), oxidation (M, W), and Trp to kynurenin (W) as variable modifications; peptide mass tolerance of 100 ppm, fragment mass tolerance of 0.5 Da; Trypsin as enzyme with a maximum of 2 missed cleavages. Compliant with minimal requirements for protein identification, no identifications purely based on the MS spectra were accepted. In addition to the requirement that the protein score had to be above the $p < 0.05$ threshold, only protein identifications supported by at least two peptides with a score above the identity threshold for individual MS/MS spectra ($p < 0.05$) specified by MASCOT were accepted. Finally, all identifications were manually validated as previously described (Printz et al., 2013) and precursors not automatically selected were fragmented to increase the sequence coverage or to identify eventual co-migrating proteins. For this validation, the list of matched peptides was compared to the MS spectrum and in cases where high-intensity peaks were not matched to the identified protein secondary database, searches were performed. If these secondary searches resulted in the identification of a second protein, the spot was excluded from biological interpretation. Performing these secondary searches, and allowing the presence of semi-tryptic peptides during the database search, resulted in the identification of processed proteins. Further validation of close-to-threshold peptide identifications were done by looking at specific spectral features. Since the different oxidized forms of tryptophan are always found together, the identification of a peptide containing one of these oxidized forms was validated by looking at the presence of peaks corresponding to the same peptide with the other oxidation products of this residue (Trp; Trp +4 Da = kynurenin, Trp +16 Da = oxidized Trp; Trp +32 Da = N-formylkynurenin). Other easy-to-recognize spectral features that were looked at include the presence of a peak corresponding to the C-terminal arginine and the presence of the neutral loss of 64 Da from peptides containing oxidized methionine. Furthermore, spectral characteristics related to the presence of the certain residues (most notably proline and aspartic acid) were analyzed (Breci et al., 2003; Paizs and Suhai, 2005). Similar for the presence of the amino acid glutamine at the N-terminus of a peptide that is generally accompanied with the same peptide at −17 Da due to the formation of pyro-glutamic acid Functional annotation for proteins of unknown function was performed using the InterProScan tool (Jones et al., 2014; Finn et al., 2017). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2014) partner repository with the dataset identifier PXD006995.

Immunoblotting

Two-dimensional (2-D) gel electrophoresis was performed using 7 cm strips (pH 4–7) as previously described, using indicated amounts of IV30, DMC or rat urine isolated leptospires. For immunoblotting, samples were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) and blocked with 5% (w/v) non-fat dried milk in phosphate-buffered saline–0.1% (v/v) Tween 20 (PBS-T). Membranes were individually incubated with indicated antisera (anti-LipL32 or anti-LipL41) at 1:2,500 in PBS-T for 1 h at room temperature, or anti-trimethyllysine or anti-acetyllysine (PTM Biolabs, IL, U.S.A.) at 1:1,000 in 1% (w/v) non-fat dried milk in PBS-T overnight at 4°C, followed by incubation with horseradish-peroxidase anti-rabbit immunoglobulin G conjugate. Bound conjugates were detected using the SuperSignal WestPico substrate (Pierce) or Clarity Western ECL substrate (BioRad) and images acquired using a UVP Biospectrum–AC w/Bio Chemi camera (Cambridge, United Kingdom) or Bio-Rad ChemiDoc MP imaging system. The specificity of the PTM-specific antibodies has been previously reported (Chu et al., 2016; Hong et al., 2016).

RESULTS

Pathogenic Leptospires Modify Protein Abundance in Response to Mammalian Host Signals

Total protein profiles of leptospires cultivated *in vitro* at 30°C (IV30), 37°C (IV37) or in DMCs were compared by 2D-DIGE over a pH range of 3–7 NL, **Figure 1**. Of 1735 protein spots aligned across 9 gels comprising 27 scans, 202 protein spots were determined to be differentially expressed ($p < 0.05$, fold change >1.25 or < −1.25) across the three conditions (**Supplementary Table 1**). When DMC-cultivated leptospires

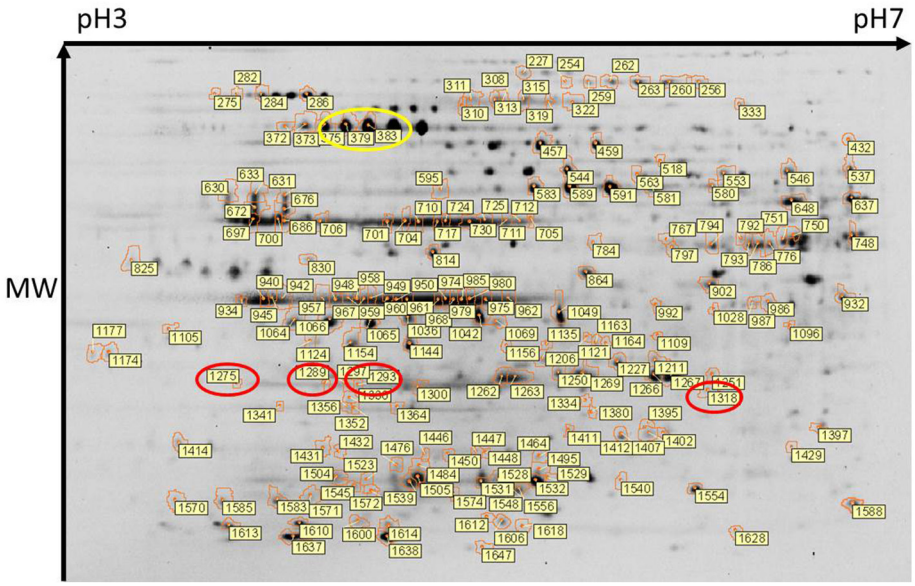


FIGURE 1 | Two-dimensional gel electrophoresis of leptospires. Composite image of protein spots detected across all 9 gels and in which 1735 protein spots were aligned. Of these, 202 protein spots were determined to be differentially expressed (DE) ($p < 0.05$). DE protein spots, and their identifier (as listed in **Supplementary Tables 1**, **2**) are indicated. Protein isoforms of the differentially expressed Loa22 and GroEL are circled in red and yellow respectively. An unmarked version of the stained gel is presented in **Supplementary Figure 1**.

were compared to IV30 leptospires, 187 spots were of different intensity ($p < 0.05$): of these, 43 were increased in DMCs whilst 144 were decreased (**Supplementary Table 1**). Similarly, when DMC-cultivated leptospires were compared to IV37-cultivated leptospires, 181 protein spots were differentially expressed ($p < 0.05$, fold change > 1.25 or < -1.25): 38 spots were increased in DMCs and 143 decreased. The majority of differentially expressed proteins are common to each group, **Figure 2**.

Comparison of IV30- and IV37-cultivated leptospires identified only 18 protein spots of significant different intensity ($p < 0.05$, fold change > 1.25 or < -1.25): seven were increased at 37°C and 11 were increased at 30°C.

Protein Identification

Spots of interest, as labeled in **Figure 1**, were excised from 2-D DIGE gel #4 for identification of proteins by mass spectrometry. Only those protein spots that had one single significant protein identification are discussed below. All protein identifications are provided in **Supplementary Tables 1**, **2**. Proteins present at a higher abundance in DMCs compared to *in vitro*-cultivated leptospires included the known virulence factor Loa22 (OmpA-like domain membrane protein), previously shown to be of higher abundance in intact motile leptospires purified from the liver of experimentally-infected guinea pigs, or in leptospires excreted from the kidney of persistently infected rats (Nally et al., 2007b, 2011); four isoforms of Loa22 (protein spots 1,318, 1,275, 1,293, and 1,289, circled in **Figure 1**, **Table 2**, and **Supplementary Tables 1**, **2**) were increased 1.9-, 1.75-, 1.58- and 1.44-fold in DMCs respectively, compared to IV30 leptospires.

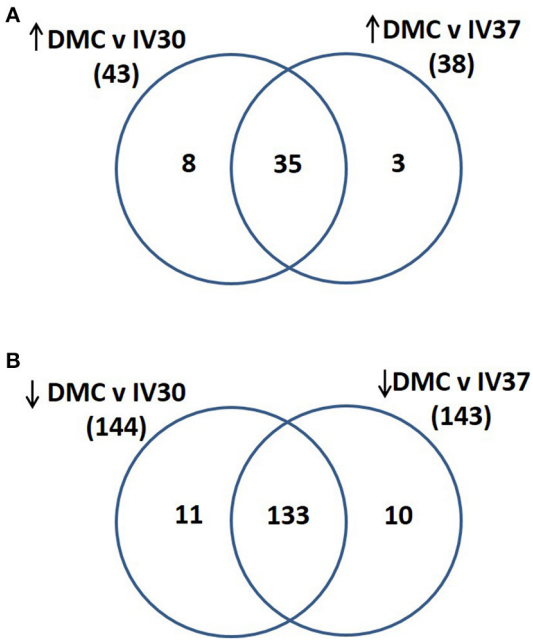


FIGURE 2 | Venn diagram illustrating the numbers of proteins that were identified as **(A)** increased in abundance in DMC compared to IV30 or IV37 leptospires or **(B)** decreased in abundance in DMC compared to IV30 or IV37 leptospires.

Similarly, the chaperone protein GroEL has also been shown to be increased in expression by leptospires excreted in urine from experimentally infected rats (Nally et al., 2011): three isoforms

TABLE 2 | Proteins more abundant ($p < 0.05$, fold > 1.25) in DMC-cultivated leptospires compared to IV30-cultivated leptospires.

Protein spot number	*p*-value	Av. ratio	GI accession number (Accession number)	Protein name (Locus tag)	InterPro scan analysis (for proteins of unknown function)
313	0.00057	7.05	gi|45602612 (AAS72087.1)	methylmalonyl-CoA mutase (LIC_20058)	
992, 987	0.000076, 0.000011	4.49, 3.71	gi|45601096 (AAS70579.1)	succinate dehydrogenase iron-sulfur subunit (LIC_12003)	
375, 383, 379	0.0012, 0.017, 0.0015	3.95, 2.59, 2.48	gi|45600451 (AAS69936.1)	GroEL (LIC_11335)	
1,028	0.0046	3.53	gi|45602037 (AAS71516.1)	LipL41 (LIC_12966)	Tetratricopeptide repeat (TPR) domain
1,164	0.013	3.09	gi|45601580 (AAS71060.1)	3-hydroxybutyryl-CoA dehydratase (LIC_12495)	
282, 284	0.00071, 0.011	2.78, 2.07	gi|45599657 (AAS69145.1)	DnaK (LIC_10524)	
1,446, 1445	0.0052, 0.045	2.4, 1.75	gi|45600006 (AAS69493.1)	putative lipoprotein (LIC_10879)	None predicted
518	0.039	2.02	gi|45601495 (AAS70976.1)	putative glutamine synthetase protein (LIC_12407)	
1,318, 1,275, 1,293, 1,289	0.0037, 0.0098, 0.026, 0.024	1.9, 1.75, 1.58, 1.44	gi|45599329 (AAS68819.1)	Loa 22 (peptidoglycan associated cytoplasmic membrane protein) (LIC_10191)	OmpA-like domain

of GroEL (protein spots 375, 383, and 379, circled in **Figure 1**, **Table 2**, and **Supplementary Tables 1**, **2**) were increased 3.95-, 2.59,- and 2.48-fold, respectively. These protein identifications serve as internal controls confirming the use of DMC-cultivated leptospires as a surrogate model for the characterization of protein expression by leptospires in response to mammalian host signals.

All the proteins that are of significant higher abundance in DMC-cultivated leptospires compared to *in vitro*-cultivated controls (IV30) are provided in **Table 2** and **Supplementary Tables 1**, **2**. Similar results are obtained when DMC leptospires are compared against IV30 or IV37 leptospires, **Figure 2**. The cobalamin-dependent methylmalonyl-CoA mutase was identified as the single protein (spot 313) that was most increased (7.05-fold change) in DMC-cultivated leptospires relative to IV30 leptospires (and increased 6.19-fold relative to IV37 leptospires). Additional metabolic enzymes increased in DMCs include succinate dehydrogenase iron-sulfur subunit (spots 992 and 987; increased 4.49- and 3.71-fold, respectively), 3-hydroxybutyryl-CoA dehydratase (spot 1,164; increased 3.09-fold) and glutamine synthetase (spot 518; increased 2.2-fold). Multiple isoforms of the chaperone protein DnaK were also increased in DMC-cultivated leptospires, as well as protein isoforms of a putative lipoprotein (LIC10879). A single protein isoform of the outer membrane lipoprotein LipL41 was identified as being increased in DMC-cultivated leptospires but this isoform (spot 1028) has an unusual mass and p*I* compared to that typically observed for LipL41 (see 7 isoforms identified below). This indicates that the protein species in spot 1,028 is a degradation product of the LipL41 protein, which corresponds in molecular mass and p*I* to an isoform of LipL41 that was previously identified in 2-D gels of leptospires cultivated at 37°C (Cullen et al., 2002).

Proteins found in higher amounts in samples from IV30- compared to DMC-cultivated leptospires are provided in **Table 3** and **Supplementary Tables 1**, **2**. Similar results are obtained when DMC leptospires are compared against IV30 or IV37 leptospires, **Figure 2**. Two isoforms of the molecular co-chaperone GroES (spots 1,638 and 1,637) were decreased in DMC-cultivated leptospires 12.47- and 9.45-fold, respectively. Two isoforms of the metabolic protein Elongation factor Tu (EF-Tu), which is hypothesized to be surface-exposed in leptospires and interact with both plasminogen and complement factor H (Wolff et al., 2013), were also decreased 10.7- and 2.81-fold, respectively. Three isoforms of peroxiredoxin (AhpC; spots 1,266, 1,263, & 1,262) were detected in lower amounts in DMC-cultivated leptospires, which is in contrast to the 5.96-fold increase in DMC for the corresponding gene transcript observed by RNAseq (Caimano et al., 2014). Consistent with lower levels of AphC protein, the levels of two isoforms of dihydrolipoamide succinyltransferase (SucB) were also decreased in abundance; this protein has been shown to interact with AphC to support antioxidant defense in *Mycobacterium tuberculosis* (Bryk et al., 2002). Proteins predicted to play a role in cell shape were found in lower amounts in DMC-cultivated leptospires and include the rod-shape determining protein MreB, cell shape determination protein LIC13483 and three isoforms of cell shape determination protein LIC12621. The expression of other

TABLE 3 | Proteins less abundant ($p < 0.05$, fold > -1.25) in DMC-cultivated leptospires compared to IV30-cultivated leptospires.

Protein spot number	*p*-value	Av. Ratio	GI Accession number (Accession number)	Protein name (Locus Tag)	InterPro scan analysis (for proteins of unknown function)
1,638, 1,637	9.3E-07, 1.1E-07	−12.5, −9.45	gi|45600452 (AAS69937.1)	GroES (LIC_11336)	
591, 583	5.6E-07, 0.00022	−10.7, −2.81	gi|45601949 (AAS71428.1)	Elongation factor Tu (LIC_12875)	
1,266, 1,263, 1,262	1.9E-07, 0.00021, 0.0099	−9.25, −2.47, −1.8	gi|45600339 (AAS69825.1)	Peroxiredoxin (LIC_11219)	
631	1.2E-06	−8.01	gi|45600988 (AAS70471.1)	Putative lipoprotein (LipL46) (LIC_11885)	None predicted
1,042, 1,049	0.000026, 0.000072	−6.47, −5.23	gi|45599498 (AAS68987.1)	Electron transport flavoprotein beta subunit (LIC_10361)	
633, 630, 672, 676	0.00014, 0.00022, 0.00026, 0.00068	−6.08, −4.32, −3.47, −3.29	gi|45602121 (AAS71599.1)	Bacterial group 3 Ig-like protein (OmpL47) (LIC_13050)	
1,610, 1,613	0.000015, 0.00021	−6.08, −4.3	gi|45600128 (AAS69614.1)	Anti-sigma factor antagonist (LIC_11004)	
1,554	0.000011	−5.02	gi|45601701 (AAS71181.1)	Cell shape determination protein (LIC_12621)	
1,066, 1,545, 1,529	0.000023, 0.00096, 0.0059	−4.81, −2.60, −1.73	gi|45600754 (AAS70238.1)	Qlp42=LipL45 (LIC_11643)	FecR protein
637	3.7E-06	−4.78	gi|45600094 (AAS69581.1)	Acyl-CoA dehydrogenase (LIC_10970)	
580, 581	6.5E-06, 2.9E-06	−4.69, −3.83	gi|45602637 (AAS72112.1)	S-adenosylhomocysteine hydrolase (LIC_20083)	
1,606	0.00012	−4.61	gi|45601539 (AAS71020.1)	Response regulator (LIC_12454)	
1,144	0.000059	−4.5	gi|45600133 (AAS69619.1)	Conserved hypothetical protein (LIC_11009)	None predicted
260, 256	0.0001, 0.0031	−4.14, −3.2	gi|45601779 (AAS71259.1)	Polyribonucleotide nucleotidyltransferase (LIC_12701)	
333	0.0015	−3.53	gi|45602455 (AAS71932.1)	Polysaccharide deacetylase (LIC_13392)	
780	0.00017	−3.42	gi|45600315 (AAS69801.1)	Putative citrate lyase (LIC_11194)	
1,174, 1,177	0.000066, 0.0047	−3.13, −1.72	gi|45600937 (AAS70420.1)	Putative lipoprotein (LIC_11834)	Fe (2+)-dicitrate sensor, transmembrane component, FecR protein
642	0.00052	−3.11	gi|45602311 (AAS71788.1)	Isocitrate dehydrogenase (LIC_13244)	
1,495	0.0071	−3.07	gi|45599867 (AAS69355.1)	Conserved hypothetical protein (LIC_10736)	Endoribonuclease L-PSP/chorismate mutase-like
1,548, 1,556	0.000052, 0.0029	−2.93, −2.07	gi|45601701 (AAS71181.1)	Cell shape determination protein (LIC_12621)	
1,250	0.000081	−2.92	gi|45600531 (AAS70016.1)	ATP-dependent Clp protease (LIC_11417)	
432	0.000084	−2.67	gi|45601716 (AAS71196.1)	Rho (LIC_12636)	
259	0.00035	−2.63	gi|45601070 (AAS70553.1)	Cyclic nucleotide binding protein (LIC_11977)	

(Continued)

TABLE 3 | Continued

Protein spot number	*p*-value	Av. Ratio	GI Accession number (Accession number)	Protein name (Locus Tag)	InterPro scan analysis (for proteins of unknown function)
962, 957, 974, 943, 960, 968, 969, 950, 979, 948, 949, 947, 975, 967, 940, 945, 959, 934, 958, 942	0.00054, 0.0016, 0.0012, 0.00081, 0.00091, 0.0017, 0.0094, 0.0027, 0.0024, 0.0019, 0.0023, 0.0017, 0.041, 0.0044, 0.0023, 0.027, 0.0038, 0.0013, 0.0054, 0.012	−2.52, −2.51, −2.48, −2.45, −2.33, −2.29, −2.23, −2.21, −2.20, −2.19, −2.17, −2.12, −2.11, −2.1, −2.09, −2.09, −2.07, −2.06, −1.98, −1.92	gi|45600468 (AAS69953.1)	LipL32 (LIC_11352)	
537, 546	0.00014, 0.00041	−2.50, −2.14	gi|45601560 (AAS71041.1)	Dihydrolipoamide succinyltransferase (LIC_12476)	
459, 227, 457	0.012, 0.03, 0.046	−2.40, −1.69, −1.56	gi|30652620 (Q72SY1.2)	ATP synthase subunit alpha (LIC_11241)	
1,612	0.00042	−2.38	gi|45602544 (AAS72021.1)	Cell shape determination protein (LIC_13483)	
814	0.0041	−2.17	gi|45600379 (AAS69864.1)	MreB (LIC_11258)	
254	0.0022	−2.13	gi|45601070 (AAS70553.1)	Cyclic nucleotide binding protein (LIC_11977)	
725, 717, 701, 711, 710, 706, 704	0.0068, 0.0035, 0.0067, 0.034, 0.011, 0.0039, 0.05	−2.07, −1.87, −1.79, −1.67, −1.61, −1.60, −1.45	gi|45602037 (AAS71516.1)	LipL41 (LIC_12966)	TPR domain
544	0.014	−2.06	gi|45600364 (AAS69849.1)	ATP synthase beta chain (LIC_11243)	
286	0.037	−2.03	gi|45599657 (AAS69145.1)	DnaK (LIC_10524)	
1,364, 1,352	0.02, 0.0081	−1.78, −1.55	gi|304570487 (AAS69491.1)	Cytochrome C/hypothetical protein (LIC10877)	
1,251	0.028	−1.71	gi|45602144 (AAS71622.1)	Transcriptional regulator (TetR family) (LIC_13073)	

proteins predicted to be involved in a wide range of cell functions was also diminished in DMC leptospires including proteins involved in RNA transcription and degradation (LIC11004, LIC13073, LIC12701, & LIC12636), amino acid metabolism (LIC20083, LIC13244, $& LIC10736), two component systems signal transduction systems (LIC12454 & LIC11194) and energy storage (LIC11241 & LIC11243).

The abundance of several known (or putative) outer membrane (OM) proteins and lipoproteins and conserved hypothetical proteins were also down in DMC-cultivated leptospires including LipL46 (LIC11885), OmpL47 (LIC13050), Qlp42/LipL45 (LIC11643), LipL32 (LIC11352), and LipL41 (LIC12966). Of note, 20 isoforms of LipL32 were all down in DMC. Similarly, the intensity of 7 spots wherein LipL41 is identified was down. Collectively, the identification of multiple isoforms of LipL32 and LipL41 derived from DMC leptospires which were all shifted in the same direction on a 2-D gel prompted us to explore the possibility that this shift was mediated

via PTM. However, analysis of MS-spectra of different spots wherein the same protein was identified failed to identify MS spectral features that could be identified as modified peptides, likely a reflection of the limited amounts of protein used in 2-D DIGE gels.

Protein Post-translational Modifications

Multiple protein species of a number of individual proteins were identified. This was exemplified by LipL32, the most abundant leptospiral outer membrane protein; 20 LipL32-isoforms were of lower abundance (< -1.25-fold, $p < 0.05$) in DMC leptospires (**Table 3** and **Supplementary Tables 1**, **2**). 2D-immunoblotting of DMC- and IV30-cultivated leptospires with LipL32-specific antiserum confirms reactivity with multiple protein isoforms, but results of immunoblot suggest that DMC- and IV30-cultivated leptospires express roughly equivalent amounts of LipL32 (**Figures 3A,B**). However, a shift in the ratio between the isoforms at different isoelectric points is observed, as indicated by an arrow in **Figures 3A,B**.

Characterization of the intact mass of LipL32 derived from purified OM vesicles confirmed that proteoforms of LipL32 can differ in their fatty acid moieties that anchor this lipoprotein into the outer membrane (i.e., lipoforms; Nally et al., 2005). More recently, studies have demonstrated that PTM occur on lysine residues of LipL32 during renal excretion compared to *in vitro*-cultivated controls (Witchell et al., 2014). Thus, we hypothesized that specific PTMs could account for, at least in part, the observed shift in p*I* of isoforms of LipL32 in DMC- compared to IV30-cultivated leptospires as detected by 2-D DIGE, and that such modifications are in response to environmental cues encountered during host infection.

To test this hypothesis, urinary derived leptospires were collected from experimentally infected rats for 2-D immunoblotting with antisera specific for the detection of defined protein PTM. Experimental infection of rats was performed with an alternative strain (strain RJ19115) to ensure that significant numbers of urinary derived leptospires could be collected for analysis by 2-D immunoblot. Immunoblotting confirmed that several protein antigens from IV30 were reactive with anti-trimethyllysine (**Figure 4A**, arrows), which were not readily detected in rat urine isolated leptospires (**Figure 4B**). Reactive antigens include several isoforms with similar mass and p*I* to that of LipL32 (**Figures 4C,D**). Additionally, immunoblotting with antibody specific for the protein PTM acetyllysine provides further evidence for differential expression of PTM by leptospires excreted in urine compared to their *in vitro*-cultivated counterpart (**Figures 5A,B**).

DISCUSSION

The transcriptome and proteome of leptospires cultivated *in vitro* has been studied extensively. However, comparative analysis of the transcription and translation of leptospiral outer membrane proteins, in response to increased temperature, identified a large number of differences in protein profile without a corresponding change in transcript levels (Lo et al., 2009). These results highlight the need to characterize the contribution of post-transcriptional regulatory pathways to mammalian host adaption by pathogenic leptospires. We have used the DMC model to comprehensively identify gene expression of leptospires *in vivo* by RNA-seq (Caimano et al., 2014). In the current study, we applied the DMC model to identify proteins that are differentially abundant in response to mammalian host signals. Our analysis confirms that pathogenic leptospires modulate protein expression in response to growth in DMC. However, and similar to previous studies comparing differential expression of gene transcription and translation in leptospires (Lo et al., 2009), none of the identified differentially abundant proteins had equivalent differentially expressed gene transcripts (based on our published RNA-seq data; Caimano et al., 2014).

Pathogenic leptospires are highly fastidious bacteria which are typically cultured in albumin enriched media at 28–30°C (Zuerner, 2005). This is in stark contrast to conditions encountered during disease transmission; leptospires adapt to and replicate in the renal tubule of a reservoir host from which they are excreted via urine to survive in suitable

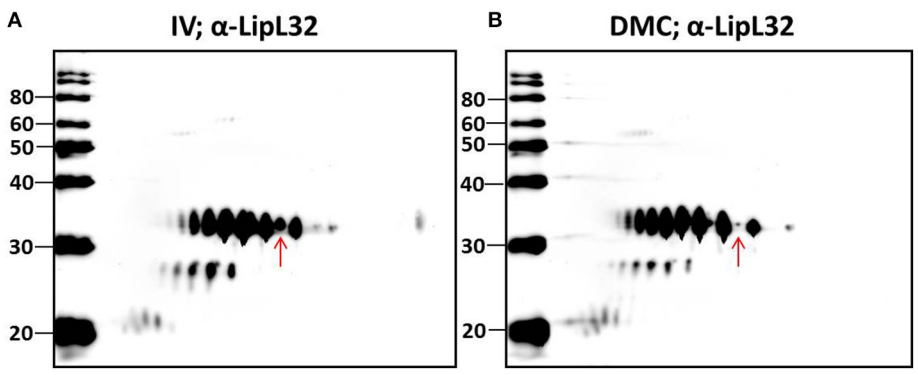


FIGURE 3 | 2-D Immunoblot analysis of **(A)** IV- and **(B)** DMC-cultivated leptospires. Approximately 10^7 IV or DMC cultivated leptospires (strain Fiocruz L1-130) were separated by 2-D gel electrophoresis and probed with antiserum specific for LipL32. Red arrow indicates the same protein isoform in each immunoblot of LipL32 when immunoblots are overlaid. Molecular mass markers (kDa) are indicated.

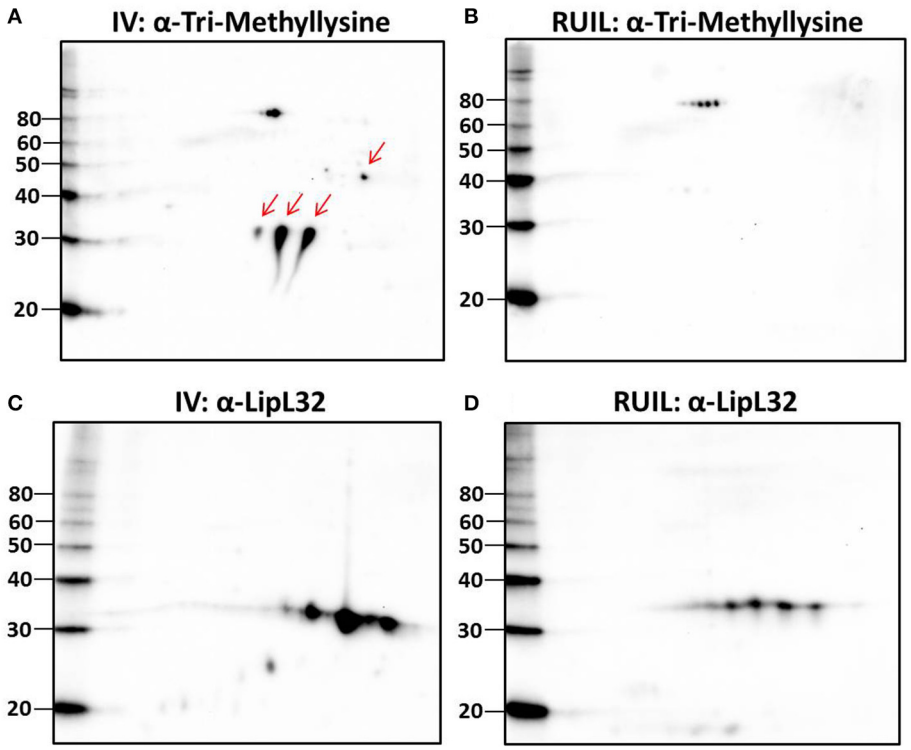


FIGURE 4 | 2-D Immunoblot analysis of IV and rat urine isolated leptospires. Approximately 2×10^7 IV30 **(A,C)** or rat urine isolated leptospires (RUIL) (Strain RJ19115) **(B,D)** were separated by 2-D gel electrophoresis (pH 4–7) and probed with antiserum specific for trimethyllysine **(A,B)** or antiserum specific for LipL32 **(C,D)**. Arrows indicate antigens reactive with anti-trimethyllysine that are expressed by IV- but not DMC-cultivated leptospires. Molecular mass markers (kDa) are indicated.

moist environments. After host invasion, they disseminate haematogenously to multiple organs (Coutinho et al., 2014), and in particular, the kidney, to establish and maintain persistent infection (Bolin and Alt, 2001; Athanazio et al., 2008). To further understand pathogenic mechanisms of leptospirosis and identify gene expression pathways and regulatory mechanisms predicted to facilitate host infection, prior studies have relied on modification of *in vitro* growth conditions to emulate conditions encountered during disease transmission and within the mammal (Matsunaga et al., 2013). Since pathogenic leptospires are not readily amenable to targeted genetic manipulation, validation of gene encoded virulence factors is generally dependent on random mutagenesis (Murray et al., 2009). Such is the case for *loa22*, the first virulence factor of leptospires identified that satisfied molecular Koch's postulates (Ristow et al., 2007). However, transcript levels of *loa22* do not change in response to temperature (Lo et al., 2006; Qin et al., 2006), osmolarity (Matsunaga et al., 2007), serum concentration (Patarakul et al., 2010) or growth in DMC (Caimano et al., 2014), but are down-regulated during interaction with macrophages (Xue et al., 2010). In contrast, the protein expression of Loa22 is significantly increased in leptospires extracted from the liver tissue of acutely infected guinea pigs (Nally et al., 2007b), and increased 2.1-fold in leptospires excreted from persistently infected rats (Monahan et al., 2008; Nally et al., 2011). In addition, levels of Loa22 are increased in leptospires cultivated in iron-depleted media and the presence of serum (Eshghi et al., 2009). Here, we report that multiple proteoforms of the Loa22 virulence factor and outer membrane protein are increased 1.9-, 1.75-, 1.58-, and 1.44-fold (spot 1,318, 1,275, 1,293, & 1,289, respectively) in response to mammalian host signals encountered by leptospires cultivated in DMC, a model system for the cultivation of leptospires *in vivo*, **Figure 1**.

Previously we have shown that the gene expression for peroxiredoxin (*ahpC*) was increased 5.96-fold in DMC-cultivated leptospires compared to IV30 leptospires. This contrasts sharply with our current findings in which three protein isoforms of AhpC were decreased 9.25-, 2.47-, and 1.8-fold in DMC compared to IV30 leptospires, and in two isoforms decreased 6.26- and 2.01-fold in DMC compared to IV37 leptospires. Recent studies comparing leptospires cultivated at 37°C compared to 30°C demonstrated that AhpC is increased 1.6-fold without a corresponding increase in gene transcript (Lo et al., 2009). Collectively, these results suggest that AhpC is subject to, and as yet uncharacterized, post-transcriptional regulation. *L. interrogans* does not encode an AhpF, the usual reducing partner for AhpC. In *M. tuberculosis*, AhpC interacts with SucB (dihydrolipoamide succinyltransferase), which was detected at lower levels in DMC leptospires compared to IV30 leptospires (−2.5 and −2.14-fold) or compared to IV37 leptospires (−2.49 and −2.01-fold).

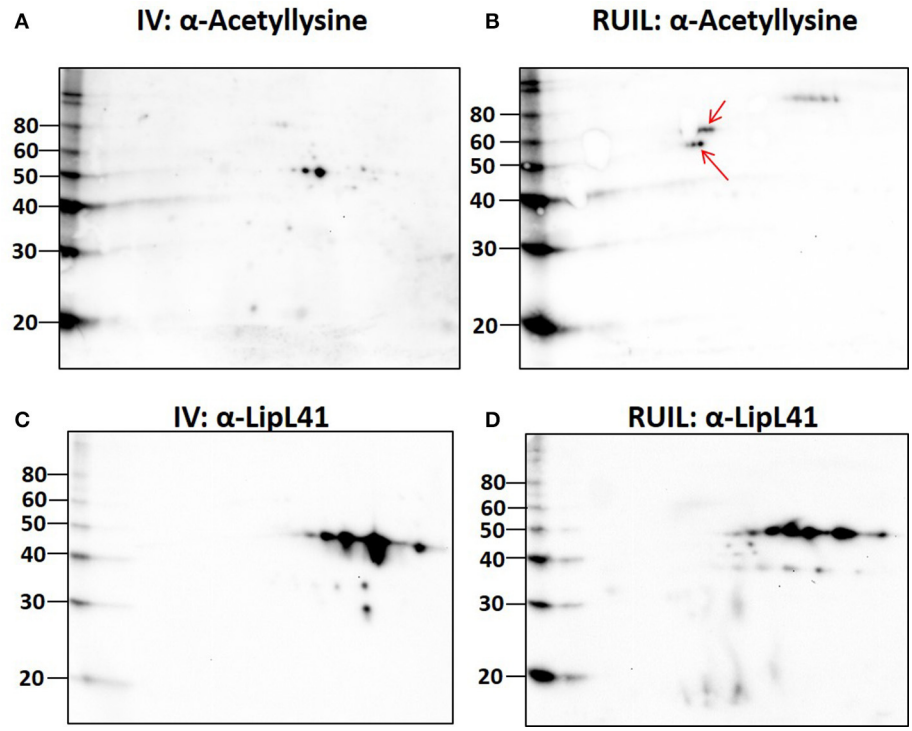


FIGURE 5 | 2-D Immunoblot analysis of *in vitro* (30°C; IV) and rat urine isolated (RUIL) leptospires. Approximately 2×10^7 or Strain RJ19115 leptospires were separated by 2-D gel electrophoresis and probed with antiserum specific for acetyllysine **(A,B)** or LipL41 **(C,D)**. Arrows indicate antigens reactive with anti-acetyllysine that are expressed by DMC- but not IV30-cultivated leptospires. Molecular mass markers (kDa) are indicated.

The cobalamin (vitamin B_{12}) dependent methylmalonyl-CoA mutase was identified in spot 313 with a fold change of 7.05 and 6.19 in DMC-cultivated leptospires compared to IV30 or IV37 leptospires, respectively. This metabolic enzyme was also identified in spots 319 and 315 which were increased 10.48- and 6.8-fold respectively in DMC leptospires compared to IV30. Recently identified small non-coding RNAs include cobalamin riboswitches which are expressed by DMC-cultivated leptospires (Ricaldi et al., 2012a; Caimano et al., 2014; Zhukova et al., 2017). These function as cis-regulatory elements in 5′-untranslated regions of vitamin B_{12}-related genes, however their function remains to be determined. Comparative analysis of 20 species of *Leptospira* predicted that only pathogenic strains make cobalamin *de novo* from L-glutamate, suggesting that this process is critical *in vivo*, and that such autotrophy allows leptospires to infect mammals in the face of vitamin B_{12} sequestration by the host (Fouts et al., 2016).

As a general trend, and similar to protein expression by leptospires cultivated at 37°C compared to 30°C (Lo et al., 2009), the abundance of many OM proteins was reduced in DMC compared to IV30 and IV37 leptospires. LipL46, a surface-exposed lipoprotein expressed during leptospiral dissemination in the mammalian host (Matsunaga et al., 2006), was downregulated 8.01- and 4.37-fold in DMC compared to IV30 and IV37 leptospires. No change in transcript levels for *lipL46* was observed in DMC or leptospires cultured at 37°C (Lo et al., 2006; Qin et al., 2006; Caimano et al., 2014), though expression of this gene has been shown to be downregulated when interacting with macrophages (Xue et al., 2010).

Four isoforms of OmpL47 (LIC13050) were 6.08-, 4.32-, 3.47-, and 3.29-fold lower in DMC leptospires compared to IV30 leptospires; similarly the same four isoforms were down 3.24-, 2.43-, 2.28-, and 2.3-fold when compared to IV37 leptospires. However, no difference was detected at the level of gene transcription by RNAseq (Caimano et al., 2014). The observed fold change of OmpL47 is similar to previous studies that demonstrated that OmpL47 is a temperature-regulated protein and of lower abundance in leptospires cultured at 37°C compared to those cultured at 30°C, and with no corresponding change in gene expression (Lo et al., 2009). In agreement with this, our analysis indicates that temperature is an important environmental cue for the expression of OmpL47 since four isoforms (spots 633, 630, 672, and 676) were of lower abundance (1.88-, 1.78-, 1.52-, and 1.43-fold, respectively) when IV37- were compared only with IV30-cultivated leptospires (**Supplementary Table 1**). Nevertheless, OmpL47 is reported to be a surface exposed protein that binds skin and elastin and is expressed during infection (Eshghi et al., 2009; Pinne et al., 2010). OmpL47 has also been detected in cell culture supernatants so it's apparent down-regulation may be due to increased levels of secretion (Zeng et al., 2013; Eshghi et al., 2015).

LipL45 was originally identified as Qlp42, the abundance of which was increased in leptospires cultivated at 37°C compared to 30°C without an apparent change in the levels of gene transcription (Nally et al., 2001a; Lo et al., 2009). In contrast to a predicted molecular mass of 39.8 kDa for LipL45/Qlp42, the actual mass was measured as 24,811 and 26,461 Da consistent with a 30 kDa doublet observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and processing of the N-terminus of the mature protein (Nally et al., 2001a, 2005; Matsunaga et al., 2002). We identified multiple isoforms of LipL45 that were detected at lower levels in DMC compared to IV30 or IV37 leptospires. InterproScan identified LipL45 as a FecR protein which is involved in the regulation of iron dicitrate transport. In the absence of citrate, FecR inactivates the probable RNA polymerase sigma factor, FecI. FecR is likely a sensor for periplasmic iron dicitrate. Gene expression levels of *lipL45* are down over 5-fold during interaction with macrophages (Xue et al., 2010). LIC11834, which encodes Lsa33, was also predicted to be a FecR protein and was similarly down in DMC leptospires. This protein binds laminin and activates plasminogen, and is predicted to be surface exposed (Domingos et al., 2012). Gene expression of *lsa33* is down-regulated −2.03-fold at physiological osmolarity compared with low osmolarity (Matsunaga et al., 2007).

Unexpectedly, 20 protein isoforms of LipL32 were uniformly identified by 2-D DIGE as differentially expressed. Similarly, 7 isoforms of LipL41 were differentially expressed. Both LipL32 and LipL41 are constitutively expressed under a wide range of *in vitro* and *in vivo* conditions, though their functions have yet to be determined (Shang et al., 1996; Haake et al., 2000). LipL32 and LipL41 are outer membrane proteins and multiple isoforms are readily observed in 2-D gels of whole or OM enriched leptospires, either by direct protein staining or immunoblotting (Nally et al., 2005, 2007b), as illustrated by the detection of LipL32 in DMC or IV30 leptospires (**Figures 3A,B**). Collectively, the apparent shift in p*I* of each of these isoforms could be explained, in part, by PTM. Multiple PTM have been detected on proteins of saprophytic and pathogenic leptospires, and on specific proteins, including OmpL32 and LipL32 which are reported to be regulated, at least in part, by exposure to mammalian host conditions encountered during infection (Eshghi et al., 2012; Witchell et al., 2014). To test this hypothesis further, leptospires were collected directly from the urine of experimentally infected rats and compared to IV30 leptospires by 2-D immunoblotting with antiserum specific for the PTM trimethyllysine and acetyllysine (**Figures 4**, **5**). The quantity of leptospires shed in the urine of experimentally infected rats is dependent on multiple factors including route of inoculation, dose, time post-infection, and strain (Athanazio et al., 2008; Bonilla-Santiago and Nally, 2011; unpublished observations). The use of an alternative strain for experimental infection of rats served to not only ensure that significant numbers of urinary derived leptospires could be collected for analysis by 2-D immunoblot, but also to ensure that our observations were relevant to more than one strain, and during actual infection as predicted by our model system for the cultivation of leptospires *in vivo* (Caimano et al., 2014). Collectively our results highlight that differences in protein modification with trimethyllysine and acetyllysine were observed between urinary vs. IV derived leptospires, indicating that these modifications were regulated, at least in part, by mammalian host signals. 2-D immunoblotting also suggested that LipL32 from IV30 leptospires contained trimethyllysine whilst urinary-derived leptospires did not; a finding which differs from the recent identification of trimethyllysine by mass spectrometry in urinary derived LipL32. The reasons for this are not yet clear and may be due to differences in modification potential of different strains of leptospires used in DMCs (Strain Fiocruz L1-130) compared to experimental infection (Strain RJ19115). In any case, a comprehensive analysis of protein structures of LipL32 as expressed during host infection is warranted. It has been hypothesized that protein PTM by leptospires may be dependent, at least partially, on elevated bacterial density (Witchell et al., 2014). Our analysis of DMC-cultivated leptospires is limited to those explanted at ∼10 days; this time-point ensures recovery of sufficient motile leptospires (up to 3×10^8 per ml) required for proteomics; this cell density in DMC corresponds to late logarithmic phase. Additional studies will be required to determine if cell density, similar to that encountered during persistent renal colonization, influences PTM. Similarly, given that DMC-cultivated leptospires are shielded from the host's immune system, it will be important to determine whether some PTMs occur in response to environmental (or physiological) stimuli vs. immune pressures encountered during persistent renal colonization.

In contrast to transcriptomic analyses which have the potential to determine the gene expression for all genes within a bacterium, gel based proteomics has inherent limitations. The genome of *L. interrogans* encodes more than 3,500 proteins (Nascimento et al., 2004), 69% of which have a theoretical p$I > 7.0$ (Nally et al., 2007a). Our analyses were limited to those proteins with a p*I* of 3–7 since previous research has demonstrated that antigens reactive with convalescent sera are expressed within this range (Kositanont et al., 2007). Under these conditions, more than 1,700 protein spots were detected across all biological replicates of DMC- or IV-cultivated leptospires. The sensitivity of gel-based proteomics is often limited to the most abundantly expressed proteins and this is reflected in those proteins we identified, given that whole leptospires were used. For example, the genes encoding DnaK, LipL32, GroEL, Elongation Factor Tu, and LipL41, are amongst the most transcribed genes in either DMC or IV leptospires (Caimano et al., 2014), and these were readily identified at the protein level. In contrast, those genes which are most differentially expressed between DMC and IV30 leptospires have (generally) an order of magnitude fewer transcripts (Caimano et al., 2014). In future work, we aim to increase sensitivity for the detection of outer membrane proteins, and their respective PTM, that interact with the host during infection; e.g., by enrichment of OM proteins with TX-114, and as required to detect the differential expression of the OM lipoprotein LipL36 in response to temperature (Nally et al., 2001b). Advantages of gel based proteomics include the

ability to identify PTM associated with specific protein isoforms, and to perform immunoblots to determine which spots are reactive with serum from exposed animals (or other ligands of interest).

Post-translational methylation and acetylation have been implicated in protein activity, resistance to proteolysis, virulence (Calder et al., 2015), phase variation of pilin, type III secretion, chemotaxis, and motility (Kort et al., 1975; Barak and Eisenbach, 2001), stress responses (Ma and Wood, 2011), and metabolism (Ouidir et al., 2015). Bacterial pathogens express OM proteins which can contain multiple PTM, and interact directly with the host during infection. Whilst generally accepted that PTM modify protein function, studies investigating the consequences of regulating these in response to host infection are limited (Cain et al., 2014; Grangeasse et al., 2015). In pathogenic leptospires, PTM of outer membrane proteins are predicted to facilitate immune evasion, and thus persistence (Witchell et al., 2014). Our results advocate that differential protein PTM, including methylation and acetylation, are regulated in response to infection. Collectively, our results highlight the need to further examine regulatory processes employed by pathogenic leptospires to adapt to the host during infection. Our analyses confirm that leptospires cultivated within DMCs, in response to mammalian host conditions, are amenable to genomic, transcriptomic and proteomic analysis and that DMC leptospires can be used to define such regulatory pathways. Finally, our results highlight the need to consider how leptospires modify their proteins with PTM, as this can influence the success of candidate vaccine and diagnostic antigens.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JN, MC. Performed the experiments: JN, AG, SP, KS, JR, MC. Contributed resources/reagents/materials/analysis tools: JN, JR, JS, AM, MC. Wrote the paper: JN. Revised the paper: JN, AG, SP, KS, JR, JS, AM, MC.

ACKNOWLEDGMENTS

We thank Stephen Hyland, Ruben Bonilla-Santiago and Richard Hornsby for technical assistance running 2-D DIGE gels, immunoblots and experimental infections of rats respectively, and support from EU COST Action FA1002 on Farm Animal Proteomics. AG was supported by a Science without Borders fellowship from the Brazilian National Council for Scientific and Technological Development. This work was partially supported by NIH grants AI-29735 (MC) and research funds generously provided by Connecticut Children's Medical Center (MC). USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00362/full#supplementary-material

Supplementary Figure 1 | Two-dimensional gel electrophoresis of leptospires. As shown in **Figure 1** but without labels.

Supplementary Table 1 | List of all differentially expressed proteins.

Supplementary Table 2 | Mass spectrometry data for all protein identifications. Search parameters are provided in the parameters sheet. The summary sheet provides all significant hits which are highlighted in green, and proteins spots with more than one significant identification are highlighted in yellow. Proteins identifications that were returned as hypothetical or unknown were subject to BLAST (Altschul et al., 1990) and these identifications are italicized.

REFERENCES

Adler, B., Lo, M., Seemann, T., and Murray, G. L. (2011). Pathogenesis of leptospirosis: the influence of genomics. *Vet. Microbiol.* 153, 73–81. doi: 10.1016/j.vetmic.2011.02.055

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Athanazio, D. A., Silva, E. F., Santos, C. S., Rocha, G. M., Vannier-Santos, M. A., McBride, A. J., et al. (2008). Rattus norvegicus as a model for persistent renal colonization by pathogenic *Leptospira interrogans*. *Acta Trop.* 105, 176–180. doi: 10.1016/jactatropica.2007.10.012

Barak, R., and Eisenbach, M. (2001). Acetylation of the response regulator, CheY, is involved in bacterial chemotaxis. *Mol. Microbiol.* 40, 731–743. doi: 10.1046/j.1365-2958.2001.02425.x

Bolin, C. A., and Alt, D. P. (2001). Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira borgpetersenii* serovar hardjo. *Am. J. Vet. Res.* 62, 995–1000. doi: 10.2460/ajvr.2001.62.995

Bonilla-Santiago, R., and Nally, J. E. (2011). Rat model of chronic leptospirosis. *Curr. Protoc. Microbiol.* Chapter 12:Unit12E 3. doi: 10.1002/97804717 29259.mc12e03s20

Breci, L. A., Tabb, D. L., Yates, J. R., and Wysocki, V. H. (2003). Cleavage N-terminal to proline: analysis of a database of peptide tandem mass spectra. *Anal. Chem.* 75, 1963–1971. doi: 10.1021/ac026359i

Bryk, R., Lima, C., Erdjument-Bromage, H., Tempst, P., and Nathan, C. (2002). Metabolic enzymes of *mycobacteria* linked to antioxidant defense by a thioredoxin-like protein. *Science* 295, 1073–1077. doi: 10.1126/science.1067798

Caimano, M. J., Sivasankaran, S. K., Allard, A., Hurley, D., Hokamp, K., Grassmann, A. A., et al. (2014). A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar copenhageni. *PLoS Pathog.* 10:e1004004. doi: 10.1371/journal.ppat.1004004

Cain, J. A., Solis, N., and Cordwell, S. J. (2014). Beyond gene expression: the impact of protein post-translational modifications in bacteria. *J. Proteomics* 97, 265–286. doi: 10.1016/j.jprot.2013.08.012

Calder, B., Soares, N. C., de Kock, E., and Blackburn, J. M. (2015). Mycobacterial proteomics: analysis of expressed proteomes and post-translational modifications to identify candidate virulence factors. *Expert Rev. Proteomics* 12, 21–35. doi: 10.1586/14789450.2015.1007046

Cao, X.-J., Dai, J., Xu, H., Nie, S., Chang, X., Hu, B.-Y., et al. (2010). High-coverage proteome analysis reveals the first insight of protein modification systems in the pathogenic spirochete *Leptospira interrogans*. *Cell Res.* 20, 197–210. doi: 10.1038/cr.2009.127

Chu, Y., Zhu, Y., Chen, Y., Li, W., Zhang, Z., Liu, D., et al. (2016). aKMT catalyzes extensive protein lysine methylation in the hyperthermophilic archaeon *Sulfolobus islandicus* but is dispensable for the growth of the organism. *Mol. Cell. Proteomics* 15, 2908–2923. doi: 10.1074/mcp.M115.057778

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

Coutinho, M. L., Matsunaga, J., Wang, L.-C., de la Peña Moctezuma, A., Lewis, M. S., Babbitt, J. T., et al. (2014). Kinetics of *Leptospira interrogans* infection in hamsters after intradermal and subcutaneous challenge. *PLoS Negl. Trop. Dis.* 8, e3307. doi: 10.1371/journal.pntd.0003307

Cullen, P. A., Cordwell, S. J., Bulach, D. M., Haake, D. A., and Adler, B. (2002). Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. *Infect. Immun.* 70, 2311–2318. doi: 10.1128/IAI.70.5.2311-2318.2002

Domingos, R. F., Vieira, M. L., Romero, E. C., Gonçales, A. P., de Morais, Z. M., Vasconcellos, S. A., et al. (2012). Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC Microbiol.* 12:50. doi: 10.1186/1471-2180-12-50

Eshghi, A., Cullen, P. A., Cowen, L., Zuerner, R. L., and Cameron, C. E. (2009). Global proteome analysis of *Leptospira interrogans*. *J. Proteome Res.* 8, 4564–4578. doi: 10.1021/pr9004597

Eshghi, A., Pappalardo, E., Hester, S., Thomas, B., Pretre, G., and Picardeau, M. (2015). Pathogenic *Leptospira interrogans* exoproteins are primarily involved in heterotrophic processes. *Infect. Immun.* 83, 3061–3073. doi: 10.1128/IAI.00427-15

Eshghi, A., Pinne, M., Haake, D. A., Zuerner, R. L., Frank, A., and Cameron, C. E. (2012). Methylation and *in vivo* expression of the surface-exposed *Leptospira interrogans* outer-membrane protein OmpL32. *Microbiology* 158(Pt 3), 622–635. doi: 10.1099/mic.0.054767-0.

Finn, R. D., Attwood, T. K., Babbitt, P. C., Bateman, A., Bork, P., Bridge, A. J., et al. (2017). InterPro in 2017—beyond protein family and domain annotations. *Nucleic Acids Res.* 45, D190–D199. doi: 10.1093/nar/gkw1107

Fouts, D. E., Matthias, M. A., Adhikarla, H., Adler, B., Amorim-Santos, L., Berg, D. E., et al. (2016). What makes a bacterial species pathogenic?: comparative genomic analysis of the genus *Leptospira*. *PLoS Negl. Trop. Dis.* 10:e0004403. doi: 10.1371/journal.pntd.0004403

Grangeasse, C., Stülke, J., and Mijakovic, I. (2015). Regulatory potential of post-translational modifications in bacteria. *Front. Microbiol.* 6:500. doi: 10.3389/fmicb.2015.00500

Grassmann, A. A., McBride, A. J. A., Nally, J. E., and Caimano, M. J. (2015). Generation of mammalian host-adapted *Leptospira interrogans* by cultivation in peritoneal dialysis membrane chamber implantation in rats. *Bio protoc.* 5:e1536. doi: 10.21769/BioProtoc.1536

Haake, D. A., Chao, G., Zuerner, R. L., Barnett, J. K., Barnett, D., Mazel, M., et al. (2000). The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect. Immun.* 68, 2276–2285. doi: 10.1128/IAI.68.4.2276-2285.2000

Hong, Y., Cao, X., Han, Q., Yuan, C., Zhang, M., Han, Y., et al. (2016). Proteome-wide analysis of lysine acetylation in adult *Schistosoma japonicum* worm. *J. Proteomics* 148, 202–212. doi: 10.1016/j.jprot.2016.08.008

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–1240. doi: 10.1093/bioinformatics/btu031

Kort, E. N., Goy, M. F., Larsen, S. H., and Adler, J. (1975). Methylation of a membrane protein involved in bacterial chemotaxis. *Proc. Nat. Acad. Sci. U.S.A.* 72, 3939–3943. doi: 10.1073/pnas.72.10.3939

Kositanont, U., Saetun, P., Krittanai, C., Doungchawee, G., Tribuddharat, C., and Thongboonkerd, V. (2007). Application of immunoproteomics to leptospirosis: towards clinical diagnostics and vaccine discovery. *Proteomics Clin. Appl.* 1, 400–409. doi: 10.1002/prca.200600805

Lo, M., Bulach, D. M., Powell, D. R., Haake, D. A., Matsunaga, J., Paustian, M. L., et al. (2006). Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. *Infect. Immun.* 74, 5848–5859. doi: 10.1128/IAI.00755-06

Lo, M., Cordwell, S. J., Bulach, D. M., and Adler, B. (2009). Comparative transcriptional and translational analysis of leptospiral outer membrane protein expression in response to temperature. *PLoS Negl. Trop. Dis.* 3:e560. doi: 10.1371/journal.pntd.0000560

Lo, M., Murray, G. L., Khoo, C. A., Haake, D. A., Zuerner, R. L., and Adler, B. (2010). Transcriptional response of *Leptospira interrogans* to iron limitation and characterization of a PerR homolog. *Infect. Immun.* 78, 4850–4859. doi: 10.1128/IAI.00435-10

Ma, Q., and Wood, T. K. (2011). Protein acetylation in prokaryotes increases stress resistance. *Biochem. Biophys. Res. Commun.* 410, 846–851. doi: 10.1016/j.bbrc.2011.06.076

Matsunaga, J., Lo, M., Bulach, D. M., Zuerner, R. L., Adler, B., and Haake, D. A. (2007). Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. *Infect. Immun.* 75, 2864–2874. doi: 10.1128/IAI.01619-06

Matsunaga, J., Schlax, P. J., and Haake, D. A. (2013). Role for cis-acting RNA sequences in the temperature-dependent expression of the multiadhesive lig proteins in *Leptospira interrogans*. *J. Bacteriol.* 195, 5092–5101. doi: 10.1128/JB.00663-13

Matsunaga, J., Werneid, K., Zuerner, R. L., Frank, A., and Haake, D. A. (2006). LipL46 is a novel surface-exposed lipoprotein expressed during leptospiral dissemination in the mammalian host. *Microbiology* 152, 3777–3786. doi: 10.1099/mic.0.29162-0

Matsunaga, J., Young, T. A., Barnett, J. K., Barnett, D., Bolin, C. A., and Haake, D. A. (2002). Novel 45-kilodalton leptospiral protein that is processed to a 31-kilodalton growth-phase-regulated peripheral membrane protein. *Infect. Immun.* 70, 323–334. doi: 10.1128/IAI.70.1.323-334.2002

McBride, A. J., Athanazio, D. A., Reis, M. G., and Ko, A. I. (2005). Leptospirosis. *Curr. Opin. Infect. Dis.* 18, 376–386. doi: 10.1097/01.qco.0000178824.05715.2c

Monahan, A. M., Callanan, J. J., and Nally, J. E. (2008). Proteomic analysis of *Leptospira interrogans* shed in urine of chronically infected hosts. *Infect. Immun.* 76, 4952–4958. doi: 10.1128/IAI.00511-08

Murray, G. L., Morel, V., Cerqueira, G. M., Croda, J., Srikram, A., Henry, R., et al. (2009). Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. *Infect. Immun.* 77, 810–816. doi: 10.1128/IAI.01293-08

Nally, J. E., Artiushin, S., and Timoney, J. F. (2001a). Molecular characterization of thermoinduced immunogenic proteins Qlp42 and Hsp15 of *Leptospira interrogans*. *Infect. Immun.* 69, 7616–7624. doi: 10.1128/IAI.69.12.7616-7624.2001

Nally, J. E., Monahan, A. M., Miller, I. S., Bonilla-Santiago, R., Souda, P., and Whitelegge, J. P. (2011). Comparative proteomic analysis of differentially expressed proteins in the urine of reservoir hosts of leptospirosis. *PLoS ONE* 6:e26046. doi: 10.1371/journal.pone.0026046

Nally, J. E., Timoney, J. F., and Stevenson, B. (2001b). Temperature-regulated protein synthesis by *Leptospira interrogans*. *Infect. Immun.* 69, 400–404. doi: 10.1128/IAI.69.1.400-404.2001

Nally, J. E., Whitelegge, J. P., Aguilera, R., Pereira, M. M., Blanco, D. R., and Lovett, M. A. (2005). Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of *Leptospira interrogans* serovar Copenhageni. *Proteomics* 5, 144–152. doi: 10.1002/pmic.200400880

Nally, J. E., Whitelegge, J. P., and Carroll, J. A. (2007b). Proteomic strategies to elucidate pathogenic mechanisms of spirochetes. *Proteomics Clin. Appl.* 1, 1185–1197. doi: 10.1002/prca.200700090

Nally, J. E., Whitelegge, J. P., Bassilian, S., Blanco, D. R., and Lovett, M. A. (2007a). Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. *Infect. Immun.* 75, 766–773. doi: 10.1128/IAI.00741-06

Nascimento, A. L., Ko, A. I., Martins, E. A., Monteiro-Vitorello, C. B., Ho, P. L., Haake, D. A., et al. (2004). Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* 186, 2164–2172. doi: 10.1128/JB.186.7.2164-2172.2004

Ouidir, T., Cosette, P., Jouenne, T., and Hardouin, J. (2015). Proteomic profiling of lysine acetylation in *Pseudomonas aeruginosa* reveals the diversity of acetylated proteins. *Proteomics* 15, 2152–2157. doi: 10.1002/pmic.201500056

Paizs, B., and Suhai, S. (2005). Fragmentation pathways of protonated peptides. *Mass Spectrom. Rev.* 24, 508–548. doi: 10.1002/mas.20024

Patarakul, K., Lo, M., and Adler, B. (2010). Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. *BMC Microbiol.* 10:31. doi: 10.1186/1471-2180-10-31

Picardeau, M. (2015). Genomics, proteomics, and genetics of *leptospira*. *Curr. Top. Microbiol. Immunol.* 387, 43–63. doi: 10.1007/978-3-662-45059-8_4

Pinne, M., Choy, H. A., and Haake, D. A. (2010). The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. *PLoS Negl. Trop. Dis.* 4:e815. doi: 10.1371/journal.pntd.0000815

Printz, B., Sergeant, K., Lutts, S., Guignard, C. D., Renaut, J., and Hausman, J.-F. (2013). From tolerance to acute metabolic deregulation: contribution of proteomics to dig into the molecular response of alder species under a polymetallic exposure. *J. Proteome Res.* 12, 5160–5179. doi: 10.1021/pr400590d

Qin, J.-H., Sheng, Y.-Y., Zhang, Z.-M., Shi, Y.-Z., He, P., Hu, B.-Y., et al. (2006). Genome-wide transcriptional analysis of temperature shift in *L. interrogans* serovar lai strain 56601. *BMC Microbiol.* 6:51. doi: 10.1186/1471-2180-6-51

Ricaldi, J. N., Fouts, D. E., Selengut, J. D., Harkins, D. M., Patra, K. P., Moreno, A., et al. (2012a). Whole genome analysis of *Leptospira licerasiae* provides insight into leptospiral evolution and pathogenicity. *PLoS Negl. Trop. Dis.* 6:e1853. doi: 10.1371/journal.pntd.0001853

Ricaldi, J., Matthias, M. A., Vinetz, J. M., and Lewis, A. L. (2012b). Expression of sialic acids and other nonulosonic acids in *Leptospira*. *BMC Microbiol.* 12:161. doi: 10.1186/1471-2180-12-161

Ristow, P., Bourhy, P., da Cruz McBride, F. W., Figueira, C. P., Huerre, M., Ave, P., et al. (2007). The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog.* 3:e97. doi: 10.1371/journal.ppat.0030097

Rojas, P., Monahan, A. M., Schuller, S., Miller, I. S., Markey, B. K., and Nally, J. E. (2010). Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 1305–1309. doi: 10.1007/s10096-010-0991-2

Schmidt, A., Beck, M., Malmström, J., Lam, H., Claassen, M., Campbell, D., et al. (2011). Absolute quantification of microbial proteomes at different states by directed mass spectrometry. *Mol. Syst. Biol.* 7, 510. doi: 10.1038/msb.2011.37

Schuller, S., Sergeant, K., Renaut, J., Callanan, J. J., Scaife, C., and Nally, J. E. (2015). Comparative proteomic analysis of lung tissue from guinea pigs with Leptospiral Pulmonary Haemorrhage Syndrome (LPHS) reveals a decrease in abundance of host proteins involved in cytoskeletal and cellular organization. *J. Proteomics* 122, 55–72. doi: 10.1016/j.jprot.2015.03.021

Shang, E. S., Summers, T. A., and Haake, D. A. (1996). Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. *Infect. Immun.* 64, 2322–2330.

Stewart, P. E., Carroll, J. A., Olano, L. R., Sturdevant, D. E., and Rosa, P. A. (2016). Multiple posttranslational modifications of *Leptospira biflexa* proteins as revealed by proteomic analysis. *Appl. Environ. Microbiol.* 82, 1183–1195. doi: 10.1128/AEM.03056-15

Vizcaíno, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Rios, D., et al. (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* 32, 223–226. doi: 10.1038/nbt.2839

Witchell, T. D., Eshghi, A., Nally, J. E., Hof, R., Boulanger, M. J., Wunder, E. A., Jr. et al. (2014). Post-translational modification of LipL32 during *Leptospira interrogans* infection. *PLoS Negl. Trop. Dis.* 8:e3280. doi: 10.1371/journal.pntd.0003280

Wolff, D. G., Castiblanco-Valencia, M. M., Abe, C. M., Monaris, D., Morais, Z. M., Souza, G. O., et al. (2013). Interaction of Leptospira elongation factor Tu with plasminogen and complement factor H: a metabolic leptospiral protein with moonlighting activities. *PLoS ONE* 8:e81818. doi: 10.1371/journal.pone.0081818

Xue, F., Dong, H., Wu, J., Wu, Z., Hu, W., Sun, A., et al. (2010). Transcriptional responses of *Leptospira interrogans* to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. *PLoS Negl. Trop. Dis.* 4:e857. doi: 10.1371/journal.pntd.0000857

Zeng, L., Zhang, Y., Zhu, Y., Yin, H., Zhuang, X., Zhu, W., et al. (2013). Extracellular proteome analysis of *Leptospira interrogans* serovar Lai. *OMICS* 17, 527–535. doi: 10.1089/omi.2013.0043

Zhukova, A., Fernandes, L. G., Hugon, P., Pappas, C. J., Sismeiro, O., Coppée, J.-Y., et al. (2017). Genome-wide transcriptional start site mapping and sRNA identification in the pathogen *Leptospira interrogans*. *Front. Cell. Infect. Microbiol.* 7:10. doi: 10.3389/fcimb.2017.00010

Zuerner, R. L. (2005). Laboratory maintenance of pathogenic *Leptospira*. *Curr. Protoc. Microbiol.* Chapter 12:Unit 12E.1. doi: 10.1002/9780471729259.mc12e01s00

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 14 March 2018
doi: 10.3389/fcimb.2018.00066

Inbred Rats as a Model to Study Persistent Renal Leptospirosis and Associated Cellular Immune Responsiveness

Jarlath E. Nally, Jennifer H. Wilson-Welder, Richard L. Hornsby, Mitchell V. Palmer and David P. Alt*

Infectious Bacterial Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA, United States

Pathogenic species of *Leptospira* cause leptospirosis, a bacterial zoonotic disease with a global distribution affecting over one million people annually. Rats are regarded as one of the most significant reservoir hosts of infection for human disease, and in the absence of clinical signs of infection, excrete large numbers of organisms in their urine. A unique biological equilibrium exists between pathogenic leptospires and reservoir hosts of infection, but surprisingly, little is known concerning the host's cellular immune response that facilitates persistent renal colonization. To address this deficiency, we established and applied an immunocompetent inbred rat model of persistent renal colonization; leptospires were detected in urine of experimentally infected rats by 3 weeks post-infection and remained positive until 8 weeks post-infection. However, there was little, if any, evidence of inflammation in colonized renal tubules. At 8 weeks post-infection, a robust antibody response was detected against lipopolysaccharide and protein outer membrane (OM) components. Purified B and T cells derived from the spleen of infected and non-infected rats proliferated in response to stimulation with 0.5 μg of OM fractions of *Leptospira*, including CD4+ T cells, which comprised 40% of proliferating cells, compared to 25% in non-infected controls. However, analysis of gene expression did not determine which immunoregulatory pathways were activated. Lymphocytes purified from the lymph node draining the site of colonization, the renal lymph node, also showed an increase in percentage of proliferating B and T cells. However, in contrast to a phenotype of 40% CD4+ T cells in the spleen, the phenotype of proliferating T cells in the renal lymph node comprised 65% CD4+ T cells. These results confirm that the renal lymph node, the local lymphoid organ, is a dominant site containing *Leptospira* reactive CD4+ T cells and highlight the need to consider the local, vs. systemic, immune responses during renal colonization infection. The use of inbred immunocompetent rats provides a novel tool to further elucidate those pathophysiological pathways that facilitate the unique biological equilibrium observed in reservoir hosts of leptospirosis.

Keywords: *Leptospira*, spirochetes, persistent renal colonization, renal lymph node, CD4+ T cells

OPEN ACCESS

Edited by:
Elsio Augusto Wunder Jr, Yale University, United States

Reviewed by:
Angela Silva Barbosa, Instituto Butantan, Brazil
Troy Bankhead, Washington State University, United States

****Correspondence:***
Jarlath E. Nally
jarlath.nally@ars.usda.gov

Received: *14 January 2018*
Accepted: *26 February 2018*
Published: *14 March 2018*

Citation:
Nally JE, Wilson-Welder JH, Hornsby RL, Palmer MV and Alt DP (2018) Inbred Rats as a Model to Study Persistent Renal Leptospirosis and Associated Cellular Immune Responsiveness. Front. Cell. Infect. Microbiol. 8:66. doi: 10.3389/fcimb.2018.00066

INTRODUCTION

Leptospirosis is a zoonotic disease of global significance caused by a unique group of bacteria (Bharti et al., 2003). Pathogenic species of *Leptospira* are excreted from colonized renal tubules of infected reservoir hosts via urine into the environment where they can survive in suitable moist conditions. Contact with urine from infected reservoir hosts, or contaminated water sources, can result in disease when pathogenic leptospires penetrate breaches of the skin, or mucosal surfaces, and disseminate haematogenously to cause a range of clinical symptoms from mild fever, to icteric Weil's disease and pulmonary hemorrhage syndrome. Mortality in these incidental hosts ranges from 10 to 70% (McBride et al., 2005). Leptospirosis is estimated to cause 1.03 million cases and 58,900 deaths each year (Torgerson et al., 2015). In developed countries, leptospirosis is primarily a recreational disease, an occupational disease of farm workers, veterinarians, and slaughter plant workers, or in returning travelers. In developing countries, it is a socioeconomic disease perpetuated by rapid urbanization, rodent infestation and transmission via contaminated water sources associated with limited infrastructures and severe weather events. Although rats are regarded as one of the most significant reservoir hosts of infection for human disease (Costa et al., 2014), many domestic animal species are also asymptomatic carriers, including dogs, cattle and pigs (Rojas et al., 2010; Ellis, 2015).

Rattus norvegicus was first recognized as a reservoir host of leptospirosis over 100 years ago, since experimental infection did not result in any clinical signs of disease, despite the persistent excretion of leptospires from kidney tissues which were subsequently lethal to guinea pigs (Ido et al., 1917). Though a unique biological equilibrium exists between pathogenic leptospires and reservoir hosts of infection, virtually nothing is known about those host-pathogen interactions that facilitate persistent renal colonization. There appears to be a specific host-parasite relationship in the Norway rat with serogroup Icterohaemorrhagiae, as compared to other serovars, since experimental infection results in persistent excretion over 220 days (Thiermann, 1981). Infected wild rats shed $>10^6$ leptospires/ml of urine and their presence in households is significantly associated with the risk of infection (Costa et al., 2014, 2015).

Experimentally infected rats persistently excrete large numbers of leptospires in urine which has allowed for the characterization of urinary derived leptospires compared to its *in vitro* cultivated counterpart (Bonilla-Santiago and Nally, 2011). Leptospires excreted from renal tubules modify their protein and antigen expression, and regulate expression of protein post-translational modifications, a function hypothesized to help evade host immune responses (Nally et al., 2005, 2011, 2017; Monahan et al., 2008; Witchell et al., 2014). Antibodies from experimentally infected rats react with a larger number of antigens expressed by *in vitro* cultivated leptospires compared to urinary derived leptospires (Monahan et al., 2008). Urine from experimentally infected rats contains host-derived biomarkers of infection (Nally et al., 2015).

In the current study, we refined the rat model of persistent renal colonization to use immunocompetent inbred rats. Leptospires colonized renal tubules by 3 weeks post-infection and were persistently excreted at 8 weeks post-infection. Despite this, no pathology was observed in renal tissues. Experimentally infected rats produced antibody against protein and lipopolysaccharide antigens of leptospires. In addition, there was a cell specific proliferative immune response. Interestingly, lymphocytes derived from spleen responded differently to those derived from the renal lymph node, demonstrating the need to study not just systemic, but localized cellular immune responses. Our results emphasize the unique biological equilibrium observed between leptospires and their respective reservoir host of infection, and provide a framework to further understand the hosts' cellular immunoregulatory pathways associated with renal colonization.

MATERIALS AND METHODS

In Vitro Cultivated Bacteria

Virulent low-passage *L. interrogans* serogroup Icterohaemorrhagiae strain RJ19115 was cultivated under standard conditions at 30°C in EMJH medium. Virulence was assessed by experimental infection of guinea pigs (Nally et al., 2004; Schuller et al., 2015).

Experimental Infection of Rats and Sample Collection

All animal experimentation was conducted in accordance with protocols as reviewed and approved by the Animal Care & Use Committee at the National Animal Disease Center, and as approved by USDA Institutional guidelines. Fifteen female Fisher 344 inbred rats (Strain F344/NHsd, Envigo) of approximately 4 weeks of age were experimentally infected with 1×10^7 low-passage *L. interrogans* strain RJ19115 by intraperitoneal injection in a final volume of 0.5 ml. From 3 to 6 weeks post-infection, urine was collected weekly from rats for enumeration of spirochetes by dark-field microscopy (DFM) as previously described (Miller, 1971). The limit of detection of leptospires by DFM is 10^5 leptospires/ml. In order to collect urine samples, rats were housed individually in a metabolism cage immediately after receiving furosemide (2–10 mg/kg) intramuscularly (Bonilla-Santiago and Nally, 2011). Nine additional rats served as non-infected controls and received 0.5 ml of culture medium.

Microscopic Agglutination Test

Serum was collected from each rat at approximately 8 weeks post-infection by cardiac puncture. The microscopic agglutination test (MAT) was performed using strain RJ19115, according to OIE guidelines at 2-fold dilutions from an initial dilution of 1:25 (Cole et al., 1973).

Pathology and Immunohistochemistry

Rat kidneys were harvested at approximately 8 weeks post-infection and immediately fixed by immersion in neutral buffered 10% formalin, processed routinely, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and

eosin (HE). Immunohistochemistry was performed on paraffin-embedded tissue sections using antiserum generated against outer membrane vesicles (OMV) of *Leptospira* species or with anti-LipL32 (Nally et al., 2004, 2017). After dewaxing, tissue sections were blocked with 10% normal goat serum in PBS for 30 mins at room temperature. Samples were incubated with anti-OMV or anti-LipL32 at 1:200 in blocking solution and incubated overnight at 4°C. After 3 × 5 min washes in PBS, samples were incubated in goat anti-rabbit IgG conjugated to AlexaFluor 546 (Invitrogen, CA) and DAPI (Invitrogen, CA) 1:3,000 in blocking solution for 60 min at room temperature in the dark. Samples were again washed in PBS before the addition of ProLong Gold anti-fade (Molecular Probes, OR) mounting media per slide and covered with a 24 × 50 mm coverslip. Samples were viewed using a Nikon Eclipse E800 and images captured using Nikon Elements Software.

Antigen Preparation and Immunoblotting

Fractionation of *L. interrogans* strain RJ19115 to enrich for outer membrane (OM) proteins was performed using Triton X-114 as previously described (Nally et al., 2001). OM enriched fractions were compared to whole leptospires by 1-D gel electrophoresis as previously described (Monahan et al., 2008). Proteins were visualized by staining with Sypro Ruby (Invitrogen, CA) and lipopolysaccharide was visualized by staining with Pro-Q Emerald 300 (Invitrogen, CA) as per manufacturer's guidelines. For immunoblotting, samples were transferred to Immobilon-P transfer membrane (Millipore, 220 Bedford, MA) and blocked overnight at 4°C with StartingBlock (TBS) blocking buffer (Thermo Scientific, CO). Membranes were individually incubated with indicated antisera (anti-LipL21, anti-LipL32 and anti-LipL41 at 1:4,000, anti-*Treponema* FlaA at 1:2,000, or a pool of sera from infected or non-infected rats at 1:1,000, in PBS-T for 1 h at room temperature), followed by incubation with horseradish-peroxidase anti-rabbit immunoglobulin G conjugate or horseradish-peroxidase anti-rat immunoglobulin G conjugate (Sigma, MO). Bound conjugates were detected using Clarity Western ECL substrate (BioRad, CA) and images acquired using a Bio-Rad ChemiDoc MP imaging system.

Lymphocyte Isolation

Spleens and renal lymph nodes were harvested from rats at approximately 8 weeks post-infection and placed in transport media (DMEM [Gibco] supplemented with 5% fetal bovine serum [FBS], 2% Pen-Strep [10,000 U/mL, Gibco], 150 μg/ml gentamicin sulfate [Sigma]). Spleens were homogenized by pressing through a 40 μm mesh cell strainer with a syringe barrel plunger in a small volume of transport media. Homogenized spleen was overlaid onto Lympholyte® -Rat (Cedarlane, Canada). Renal lymph nodes were homogenized by gently mashing using the frosted end of two acid-washed autoclaved glass microscope slides, resuspended in PBS and overlaid onto Lympholyte® -Rat. Renal lymph nodes from 3 infected or non-infected animals were pooled together, while spleens were processed individually. After centrifugation, the lymphocyte cell layer was recovered and washed once with PBS. Red blood cells were then lysed by ammonium-chloride-potassium (ACK) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.01 mM Na_2EDTA) for 90 s. Cells were passed through a 40 μm cell filter, washed by centrifugation, resuspended in PBS containing DNase (1 mg/mL, Sigma D25) for 10 min and washed again. Washed cells were resuspended in PBS and overlaid onto 5 ml FBS for a final wash. Cells were counted on a hemocytometer after staining with trypan blue. Cells were labeled with 10 nM Cell-Trace Violet (Molecular Probes) following manufacturer's recommendations and resuspended at a final concentration of 5×10^6 per ml in DMEM media supplemented with 1% sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), 1% non-essential amino acids (Gibco), 10% FBS, 5×10^{-3} mM β-mercaptoethanol, 1% Pen-Strep (10,000 U/mL, Gibco) and 100 μg/ml gentamicin sulfate (Sigma). Cells were cultured in 96 well round bottom plates (5×10^5 cells/well) in the presence of *L. interrogans* strain RJ19115 OM antigen at 0.5 μg/ml. This dose was experimentally determined to be optimal for stimulation. Concanavalin A (1 μg/ml) was included as a positive control stimulant, and media only was used as no stimulant/negative control. Cells were cultured for 4 days at 39°C with 5% CO_2.

Flow Cytometry and Statistical Analysis

At 4 days post-stimulation in culture, cells were harvested by centrifugation, and labeled with live/dead discriminator dye (Zombie Yellow, Biolegend, CA) followed by antibodies to cell surface markers for CD3, CD4, CD8b, gamma-delta T cell receptor, NK T cell marker (CD161a), B220, and CD19. Primary antibodies, secondary antibodies, dilutions and suppliers are provided in **Supplementary Table 1**. Following labeling, cells were fixed (Stabilizing Fixative 3X, BD Bioscience, CA) and data collected using BD LSRII Flow Cytometer. Data analysis for cell phenotype was performed using FlowJo software with 2,000 cells within the live gate required for analysis. An example of gating strategy is provided in **Supplementary Figure 1**. Proliferation was indicated by a decrease in fluorescence intensity of cell membrane proliferation tracking dye as compared to no stimulation wells. Phenotype of proliferating subsets were determined with gate restrictions being set using fluorescence-minus-one. Data was further analyzed for statistical significance using GraphPad Prism 7 software fitting 2-way ANOVA with Sidak's multiple comparisons post-test, comparing within groups (Control or Infected) effect of well stimulation to no stimulation wells and between groups for a given well treatment. Mean percentages were significant if $p \leq 0.05$. Statistical significance for cell phenotype between groups was determined by multiple *t*-tests using the Holm-Sidak method with alpha = 0.05.

Gene Expression Arrays and Statistical Analysis

Spleens were harvested from rats at approximately 8 weeks post-infection and a portion flash frozen on dry ice. Samples were stored at −80°C until RNA extraction using the RNeasy RNA kit (Qiagen). Purified RNA was treated with Ambion Turbo Free DNase (Thermo-Fisher) and sample quality checked using the BioAnalyzer 2,100–RNA Chip Nano 6,000 (Agilent, CA). Two microgram of RNA was used per 150 μl cDNA synthesis reaction (Invitrogen Superscript IV First Strand Synthesis

kit) for each animal. cDNA amounts were normalized and pooled such that 20 ng from three infected or non-infected animals provided 60 ng per each well of a BioRad Prime PCR custom array PCR assay plate. Genes assayed, relevant gene identification information, and primer sequence as supplied by the manufacturer are supplied in **Supplementary Table 2**. RT-PCR assays were performed using SSOAdvanced Universal SYBR Green Supermix (BioRad) on 384 CFX C1000 Touch Thermal Cycler (BioRad). QRT-PCR analysis was carried out using BioRad CFX Manager Software using Single Threshold and the relative gene expression levels were calculated using comparative Ct ($\Delta\Delta$Ct) method, normalized to the expression of 2 housekeeper genes (*actb*, *b2m*). Replicate wells with Ct standard deviations >0.5 were removed from further analysis. Gene expression was calculated as $2^{-\Delta\Delta Ct}$ and then Log_2 transformed using GraphPad Prism 7 for statistical analysis. Statistical significance was determined by multiple *t*-tests using the Holm-Sidak method with alpha = 0.05. An expression ratio of 1.5 was chosen as difference from control (non-infected).

RESULTS

Persistent Excretion of Leptospires

All experimentally infected rats were positive excretion of leptospires in urine, as detected by dark-field microscopy (DFM), by 3 weeks post-infection, **Table 1**. Numbers of leptospires in urine ranged from 1×10^5 leptospires/ml (the lowest limit of detection by DFM) up to 1×10^7 leptospires/ml. Kidneys from all experimentally infected rats were culture positive demonstrating persistent renal colonization until the end of the experiment. All infected rats displayed similar weight increases compared to non-infected controls (data not shown).

Pathology and Immunohistochemistry

All kidneys from experimentally infected rats were positive by immunohistochemistry using antibody specific for outer membrane vesicles (OMV) or anti-LipL32, **Figure 1**. In general, there was little, if any, evidence of inflammation. Direct comparison of areas positive for leptospires by immunofluorescence with their HE counterparts did not identify corresponding areas of inflammation. Occasionally, mild small foci of low numbers of interstitial lymphocytic infiltrates were observed in both infected and non-infected rats; none of these areas were positive for leptospires.

Humoral Response

All experimentally infected rats had a positive MAT titer that ranged from 1:400 to 1:1,600 indicating a strong antibody response against leptospiral lipopolysaccharide, **Table 1**. A strong antibody response against leptospiral proteins was also evidenced by immunoblot, **Figure 2A**. Non-infected rats were negative by MAT (titer <1:25) and immunoblot, **Figure 2B**.

Cellular Response

Purified lymphocytes were stimulated with a fraction of *Leptospira* enriched for outer membrane (OM) components. The interaction of OM proteins with the host during infection is indicated by a positive immunoblot, **Figure 2A**, compared to non-infected controls, **Figure 2B**. The protein content of the OM enriched fraction compared to the total protein profile of leptospires indicates those proteins that partitioned to the OM fraction, **Figure 2C**. The OM fraction contains lipopolysaccharide, **Figure 2D**, as well as a number of well characterized OM proteins including LipL21, LipL32, and LipL41, **Figures 2E–G**, respectively. As expected for the periplasmic FlaA, it was detected in minimal amounts in the OM fraction compared to whole leptospires, **Figure 2H**.

To examine relative numbers and phenotypes of antigen responsive cells, lymphocytes were isolated from chronically infected inbred rats. Purified lymphocytes derived from the spleen of 15 infected and nine non-infected rats proliferated in response to stimulation with 0.5 μg of OM fractions of *Leptospira*. Both infected and control animals showed a statistically significant ($p < 0.001$) increase in percentage of proliferating B cells (CD3−, B220+, or CD19+) and T cells (CD3+) when cultured in the presence of *Leptospira* OM fraction compared to lymphocytes cultured with media alone (No Stim), **Figures 3A,B**. Additionally, a significant ($p < 0.001$) increase was observed in the percentage of proliferating B-cells and T-cells isolated from spleens of infected rats when compared to non-infected controls. The phenotype of proliferating T cells stimulated with 0.5 μg OM fraction from infected animals comprised 40% CD4+ (standard deviation [SD] 8), 26% CD8b+ (SD 12), 39% NK/CD161a+ (SD 8), and 24% γδ-TCR+ (SD 7), **Figure 3C**. The phenotype of proliferating T cells stimulated with 0.5 μg OM fraction from control animals comprised 25% CD4+ (SD 9), 28% CD8b+ (SD 12), 48% NK/CD161a+ (SD 12), and 23% γδ-TCR+ (SD 7), **Figure 3C**. There was a statistical difference ($p = 0.0013$) in the percentage of proliferating CD4+ population between control and infected animals, indicating the presence and proliferation of *Leptospira* specific reactive CD4+ T cells in the spleen of persistently infected rats.

In order to determine effector T cell functions or specific immune pathways that may have been induced by chronic *Leptospira* infection, gene expression for T-cell activation was analyzed in spleens of 15 infected and nine control rats. A custom T-cell activation panel was chosen to represent various T cell pathways including Th1, Th2, Th17, and Treg (**Supplementary Table 2**). As shown in **Figure 4**, none of the genes assayed were significantly differentially expressed ($p < 0.05$) above an expression threshold of 1.5.

Given that the host response may be more localized to the site of colonization by *Leptospira*, antigen responsive lymphocytes derived from the renal lymph node (RLN) of 15 experimentally infected rats, and nine non-infected controls, were assayed. Lymphocytes were isolated from the RLN, pooled into groups of three animals, and stimulated with purified OM fraction from *Leptospira*. Both infected and control rats showed a significant increase ($p < 0.005$) in percentage of proliferating B cells (CD3−, CD19+, or B220+) when stimulated with 0.5 μg/ml OM antigen compared to no stimulant controls, **Figure 5A**, but no difference was observed between infected and control animals. Lymphocytes from infected RLN showed a significant increase in proliferating T cells (CD3+) compared to control RLN

TABLE 1 | Experimentally infected rats were positive for persistent renal leptospirosis.

Animal #	Week 3	Week 4	Week 5	Week 6	Culture	MAT
1	1.00E + 05	1.00E + 05	1.00E + 05	ND	+	1:800
2	5.00E + 06	1.00E + 07	5.00E + 06	3.00E + 06	+	1:1,600
3	1.00E + 06	1.00E + 06	3.00E + 06	3.00E + 06	+	1:1,600
4	5.00E + 06	8.00E + 06	3.00E + 06	4.00E + 06	+	1:1,600
5	3.00E + 06	2.00E + 06	2.00E + 06	3.00E + 06	+	1:800
6	1.00E + 05	1.00E + 05	1.00E + 05	ND	+	1:400
7	1.00E + 06	2.00E + 05	1.00E + 06	1.00E + 06	+	1:400
8	1.00E + 06	5.00E + 05	2.00E + 05	ND	+	1:800
9	3.00E + 06	3.00E + 06	1.00E + 06	4.00E + 06	+	1:800
10	5.00E + 06	2.00E + 06	1.00E + 06	1.00E + 06	+	1:1,600
11	7.00E + 05	1.00E + 06	1.00E + 06	1.00E + 06	+	1:400
12	1.00E + 07	5.00E + 06	2.00E + 06	1.00E + 06	+	1:1,600
13	1.00E + 06	2.00E + 05	2.00E + 06	3.00E + 06	+	1:800
14	1.00E + 05	2.00E + 05	1.00E + 05	ND	+	1:400
15	2.00E + 05	1.00E + 05	3.00E + 05	ND	+	1:400
Mean	2.56E + 06	2.36E + 06	1.53E + 06	2.18E + 06		

Numbers of leptospires excreted per ml of urine, as detected by dark-field microscopy, at 3, 4, 5, and 6 weeks post-infection. At 8 weeks, all rats were kidney culture positive and MAT positive at indicated titer.
ND, Not detected.

($p = 0.007$) and no stimulant controls ($p = 0.0069$; **Figure 5B**). The phenotype of proliferating T cells stimulated with 0.5 μg OM fraction in RLN from infected animals comprised 65% CD4+ (SD 1), 13% CD8b+ (SD 3), 14% NK/CD161a+ (SD 5), and 14% γδ-TCR+ (SD 1), **Figure 5C**. The phenotype of proliferating T cells stimulated with 0.5 μg OM fraction in RLN from control animals comprised 30% CD4+ (SD, 4), 24% CD8b+ (SD 3), 33% NK/CD161a+ (SD 1), and 30% γδ-TCR+ (SD 1), **Figure 5C**. There was a statistical difference in all the T cell subsets between control and infected animals ($p < 0.001$), with the greatest difference being between the proportion of CD4+ subsets. These results confirm that the RLN, the local lymphoid organ, is the site containing the majority of the *Leptospira* reactive CD4+ T cells.

DISCUSSION

Host-pathogen relationships have evolved over millennia and range from asymptomatic, chronic and persistent carriage in some hosts compared to acute, fulminant disease in others. The unique biological equilibrium that exists between reservoir hosts of infection and bacterial pathogens is facilitated in part by the ability of the pathogen to express appropriate virulence factors that maintain infection, yet minimize detection and subsequent clearance by host immune responses. *Rattus norvegicus* is one of the most important reservoir hosts for the persistent dissemination and transmission of pathogenic leptospires to human populations throughout the world. Wild rats can routinely excrete $>10^6$ leptospires/ml of urine, yet they also show evidence of an antibody response against lipopolysaccharide, and a cellular immune response as evidenced by interstitial nephritis (Tucunduva de Faria et al., 2007; Costa et al., 2015). In this manuscript, an immunocompetent inbred rat model of leptospirosis was developed to specifically address the host cellular immune responses associated with persistent renal infection.

Acute leptospirosis in humans and domestic animals is generally described as a biphasic disease (Haake and Levett, 2015). Phase one comprises a leptospiraemia during which leptospires disseminate throughout the host and thus cause a wide range of clinical symptoms, ranging from fever to pulmonary hemorrhage. The second convalescent phase is associated with the appearance of agglutinating antibodies which are believed to mediate clearance of leptospires. At this time, leptospires are no longer detected in blood but may be detected in urine. Urinary excretion in incidental hosts tends to be short-lived. A similar profile is observed in laboratory animal models of acute leptospirosis, including hamsters and guinea pigs, whereby an acute lethal disease is associated with dissemination of leptospires throughout the host; this is accompanied by significant weight loss and pathology in a range of tissues which can include lung, liver and kidney (Nally et al., 2004; Coutinho et al., 2011; Wunder et al., 2016). Experimental infection of *Rattus norvegicus* is also associated with an initial leptospiraemic phase during which leptospires are detected in liver, kidney and skeletal muscle but, in contrast to acute infection, no underlying histopathology, or weight loss, is observed (Athanazio et al., 2008; Monahan et al., 2008). By day 9 post-infection, leptospires are cleared from all organs except the kidney, and experimentally infected rats can become persistent urinary shedders of leptospires for months (Thiermann, 1981; Rojas et al., 2010; Bonilla-Santiago and Nally, 2011). Similarly, we now report that inbred immunocompetent *Rattus norvegicus* (Fisher 344) are susceptible to infection with *L. interrogans* serogroup Icterohaemorrhagiae as characterized by persistent renal colonization and urinary shedding for at least 8 weeks post-infection in the absence of pathology or weight loss.

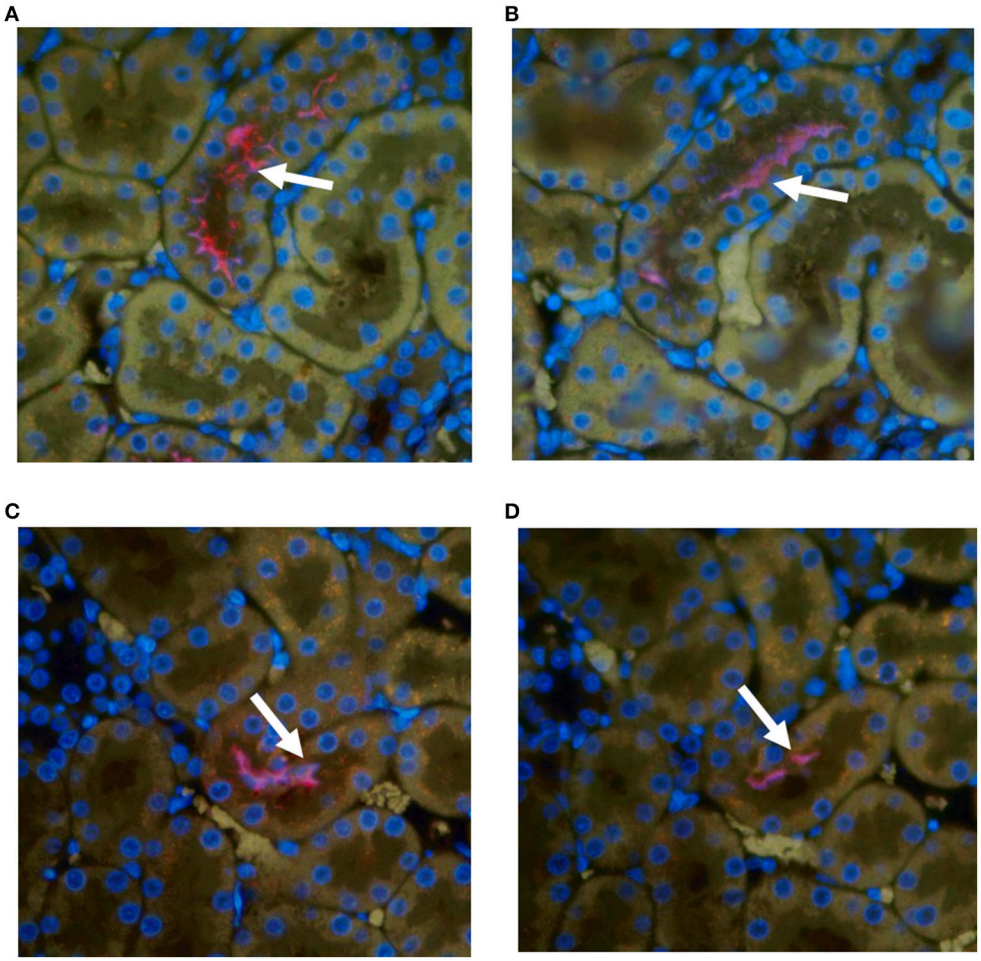


FIGURE 1 | Representative immunohistochemical staining of kidneys from experimentally infected rats at 8 weeks post-infection with anti-OMV **(A,C)** or anti-LipL32 **(B,D)**. Arrows indicate positive renal tubules. Original magnification × 400.

Urinary excretion of leptospires at 8 weeks post-infection was evident despite the detection of a robust antibody response specific for both lipopolysaccharide and protein antigens (**Table 1** and **Figure 2**). Antibody specific for the O-antigen of leptospiral polysaccharide is considered protective (Challa et al., 2011). The production of antibody was supported by the detection of proliferating B cells in the spleen of infected rats when stimulated with OM antigen. Experimental models using B cell deficient mice, suffering acute infection, have shown that B cells are a crucial lymphocyte subset responsible for the clearance of leptospires from liver and peripheral tissues (Chassin et al., 2009). Polymeric bacterial antigens, such as LPS, can bind directly to B cell receptors and induce B cell activation, even in the absence of the cognate T cell (Murphy and Weaver, 2016). *Leptospira* specific antibody may be found in the urine (Nally et al., 2011), however, the lack of innate or other cell-types, which interact with pathogen bound antibody for an effector function, within the undamaged renal tubule makes the antibody of little consequence to the pathogen (Lu et al., 2018). Antigen-specific proliferating CD3+ T cells were evidenced in spleen and included CD4+, CD8+, NK, and ϒδ-TCR+. The proportion of CD4+ CD3+ antigen stimulated proliferating cells increased from 25 to 40% in those spleens derived from infected rats compared to non-infected controls (**Figure 3**). In order to identify those immunoregulatory pathways involved in cellular activation, gene expression profiles of spleen tissue from infected or non-infected control rats were assayed with a custom gene expression array that included genes previously shown to regulate T cell pathways, including Th1, Th2, Treg, and Th17 (**Supplementary Table 2**; Belkaid and Rouse, 2005; Iwakura et al., 2008; Gasteiger and Rudensky, 2014; Godfrey et al., 2015). No differences in gene expression values were observed in the spleen from infected and control animals (**Figure 4**). This may be a result of multiple factors including (1) the lack of pathology observed in the kidney despite the presence of leptospires which suggests a limited activation of the host response, (2) the limited number of genes assayed (3) the low numbers of T cells induced in the spleen in this model of infection, or (4) the selected time point post-infection for analysis. These observations are in keeping with an increase in levels of gene expression for both IL17a, an

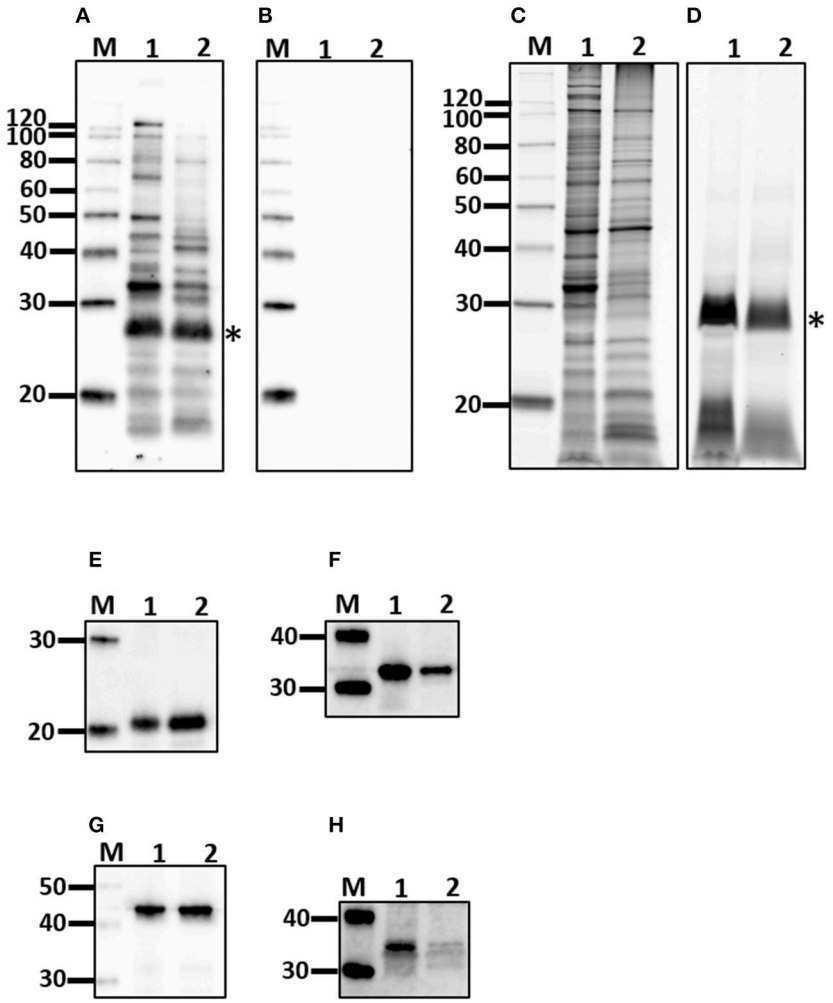


FIGURE 2 | Characterization of outer membrane (OM) fraction. Antigen derived from whole cells (1) was compared to the OM fraction (2) by immunoblotting with sera from experimentally infected rats **(A)** or non-infected controls **(B)** and for protein **(C)**, and lipopolysaccharide content **(D)**, as well as the presence of LipL21 **(E)**, LipL32 **(F)**, LipL41 **(G)**, and FlaA **(H)**. *Indicates O-antigen. Molecular mass markers (M) are indicated.

indicator of pro-inflammatory immune responses and FoxP3, a transcription factor associated with the development of T-regulatory cells, in infected rat spleens that failed to reach statistical levels of significance (**Figure 4**). Analysis of differential gene expression at earlier time points post-infection may provide additional insights.

Given that the presence of leptospires in the rat was limited to renal tissues, lymphocytes were also purified from the lymph node draining the site of colonization, the renal lymph nodes. As with the spleen, proliferating B cells were readily detected in stimulated cells, though similar profiles were observed whether they were derived from infected or non-infected animals. However, there was also a readily detected antigen specific increase in proliferating CD3+ T-cells from the renal lymph node of infected rats compared to non-infected rats (**Figure 5**); the percentage of antigen proliferating CD4+ T cells increased from 30 to 65% compared to other cell types; CD8+ T cells decreased from 24 to 13%, NK cells from 33 to 14% and ϒδ-TCR+ from 30 to 14%. Additional phenotypic markers and/or cytokine analysis will be required to determine whether the memory phenotype or functionality (Th1-Th2-Th17) of these antigen-reactive CD4+ T cells in the spleen are similar to those in the renal lymph nodes of chronically infected rats.

A predominant lesion in naturally infected chronic rat leptospirosis is diffuse interstitial nephritis (Sterling and Thiermann, 1981; Tucunduva de Faria et al., 2007; Monahan et al., 2009; Agudelo-Flórez et al., 2013). In rats experimentally infected with 10^8 *L. interrogans* serovar Copenhageni, interstitial nephritis was detected in 33% of rats by 4 weeks post-infection (Tucunduva de Faria et al., 2007); by 2 months, this number increased to 71.4% thus highlighting the dynamic nature of the cellular immune response. No interstitial nephritis was observed during our study at 8 weeks-post infection though this may be due to the lower dose of 10^7 leptospires used in

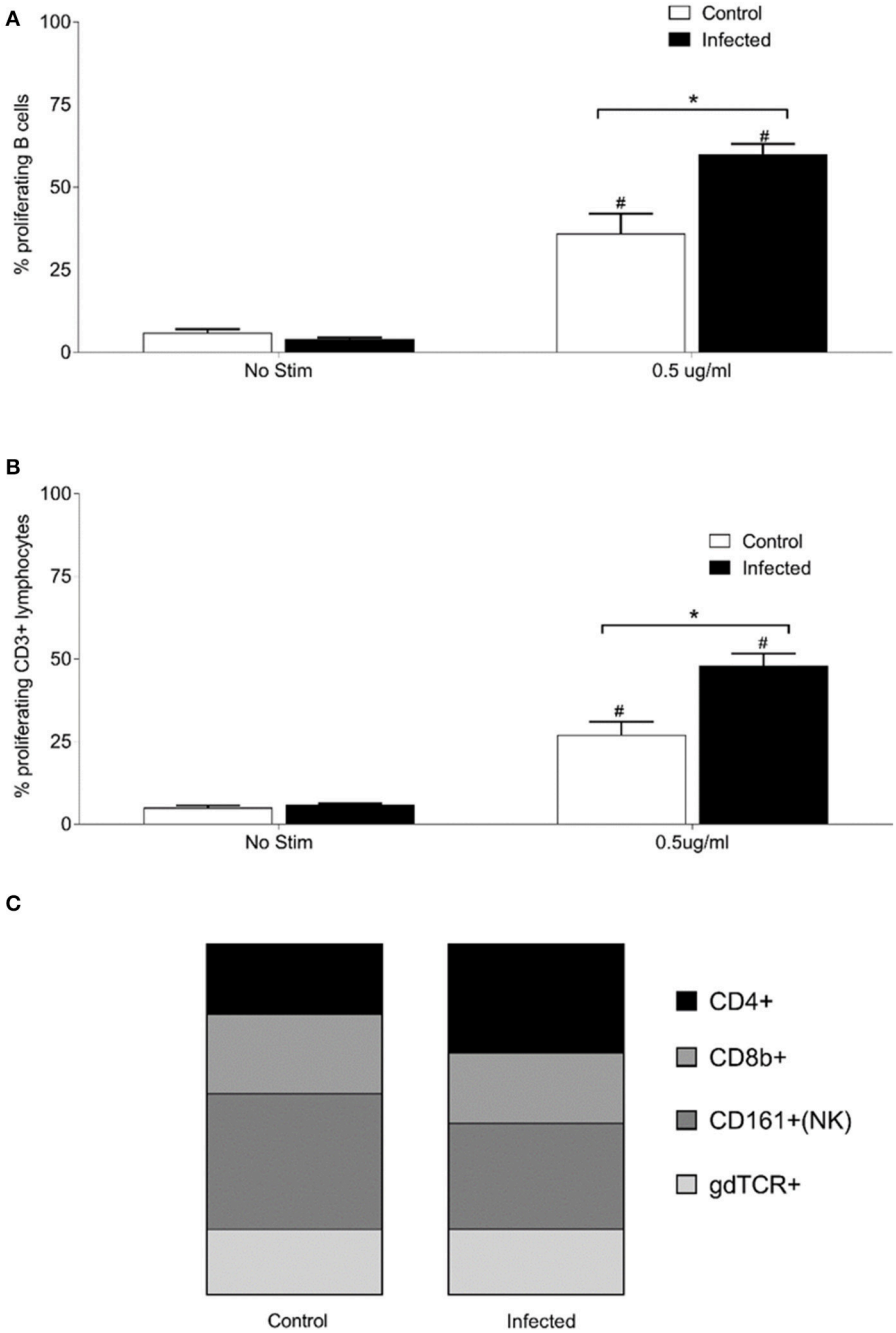


FIGURE 3 | Proliferative responses and cellular phenotypes of lymphocytes isolated from the spleen of 15 infected and nine control rats. Percentage of proliferating B cells (CD3−, CD19+, or B220+) **(A)**, percentage of proliferating T cells (CD3+) **(B)** and phenotype by proportion of proliferating CD3+ cells stimulated with 0.5 μg/ml OM antigen **(C)**. *indicates statistical significance between infected and control animals; #Indicates statistical significance between OM antigen stimulated wells and no stimulant (No Stim) wells within the same group.

our study. However, interstitial nephritis has been observed at 160 days post-infection in rats experimentally infected with a lower dose of 10^6 leptospires. At this time, a lymphocyte-rich inflammatory infiltrate in association with leptospires in infected kidney sections was apparent; however, it was also apparent that leptospiral organisms were also detected within tubules devoid of any immune response. It remains unclear if such responses can ultimately be successful enough to remove organisms and eliminate renal excretion, or whether leptospires can continue to evade detection and reactivity as hypothesized by differential expression of antigens (Nally et al., 2007, 2011, 2017; Monahan et al., 2008; Witchell et al., 2014).

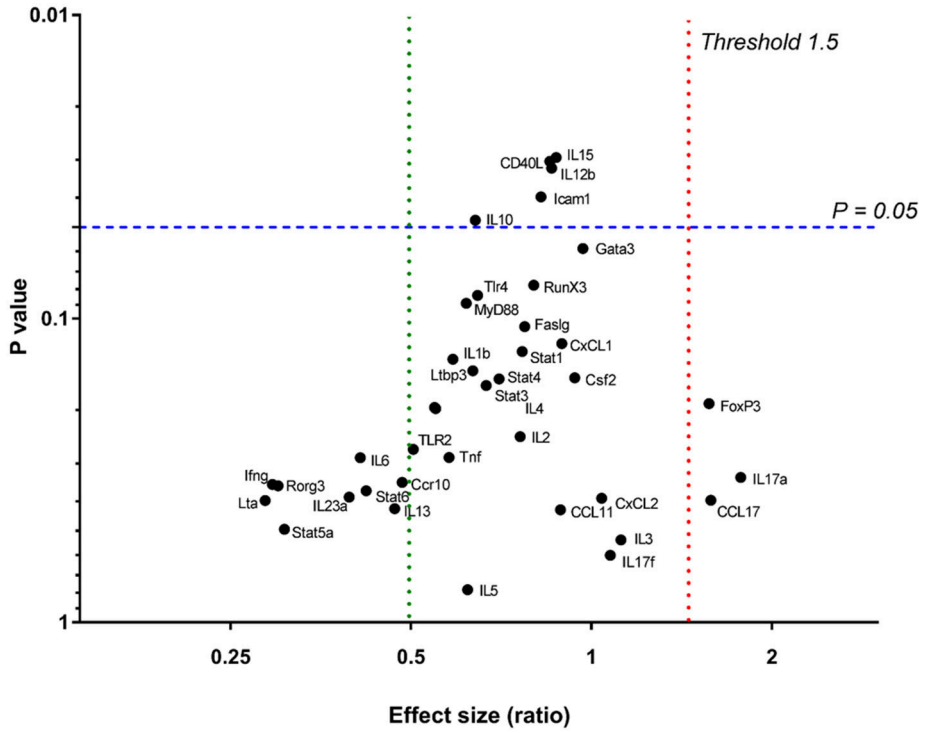


FIGURE 4 | Relative gene expression in spleens of 15 infected vs. nine control rats. $P = 0.05$ is signified by a blue dashed line. The upper expression threshold (upregulated genes) (Effect size [ratio]) of 1.5 is signified by a red dotted line and lower expression (downregulated) threshold of 0.5, by a green dotted line.

At 8 weeks post-infection, all rats had a positive MAT titre ranging from 1:400 to 1:1600. This differs to experimentally infected rats that were MAT negative at 160 days post-infection (Nally et al., 2015). This discordance is likely due to waning titers over time since experimentally infected rats were exposed to infection only once, compared to wild type rats, which appear to maintain titers, and are routinely exposed via multiple routes (Costa et al., 2015; Minter et al., 2017). The MAT is typically used to diagnose acute disease; in the absence of clinical signs of infection, it only serves to indicate exposure, and is of limited use to detect domestic animals that are acting as reservoir hosts of infection. Cattle, acting as reservoir hosts for serovar Hardjo, are routinely MAT negative (Miller et al., 1991; Libonati et al., 2017). The leading cause of bovine leptospirosis is infection with serovar Hardjo, including both members of *L. interrogans* and *L. borgpetersenii*. Most bovine infections are inapparent clinically, despite the potential shedding of viable *Leptospira* for extended periods of time. Isolation, although successful from bovine reproductive tissues, is most frequently accomplished as seen here with the rat, from either the kidney or from urine. Although interstitial nephritis can be detected in the kidneys of cattle experimentally infected with *L. borgpetersenii* serovar Hardjo, lesions produced are usually minimal in scope and limited to focal capsular depressions or areas of pallor. Histologically, these foci may show evidence of tubular and glomerular damage with inflammatory infiltrates and fibrosis (Bolin and Alt, 2001; Zuerner et al., 2011). Protective vaccine-induced responses in cattle tend to favor a Th-1 immune response involving both CD4+ T cells and ϒδ-T cells (Naiman et al., 2002).

Studies on the cell-mediated immune response to leptospirosis have been limited to humans and small animal models with acute symptoms. Hamsters and humans, which exhibit acute disease, express high levels of pro-inflammatory cytokines IL-6 and TNFα in infected tissues or from PBMC (Matsui et al., 2011; Volz et al., 2015). Increased levels of IL-10, while also highly expressed in infected hamsters, were associated with milder disease in humans (Raffray et al., 2015). Humans with higher bacterial burdens had decreased numbers of circulating ϒδ-T cells (Raffray et al., 2015). However, a C3H/HeJ mouse model of nonlethal acute disease, induced a more Th2-mediated CD4+ effector response (Richer et al., 2015). Much of the discrepancy in conflicting reports comes from trying to draw parallels between not just different species, but different types of infection: mild to sublethal to acute lethal disease. The rat, an asymptomatic reservoir host, does produce antibody and a robust cellular response at the draining lymph node, despite an apparent lack of inflammatory responses. While a Th1 vs. a Th2 type response was not elucidated in this current study, it may be that chronic leptospirosis in the reservoir host is similar to that of syphilis and starts out as a Th1 response with robust antibody; with time, and as the disease changes tissue tropism, the immune response shifts to a more Th2-like response as the pathogen and host reach biological equilibrium (Fitzgerald, 1992).

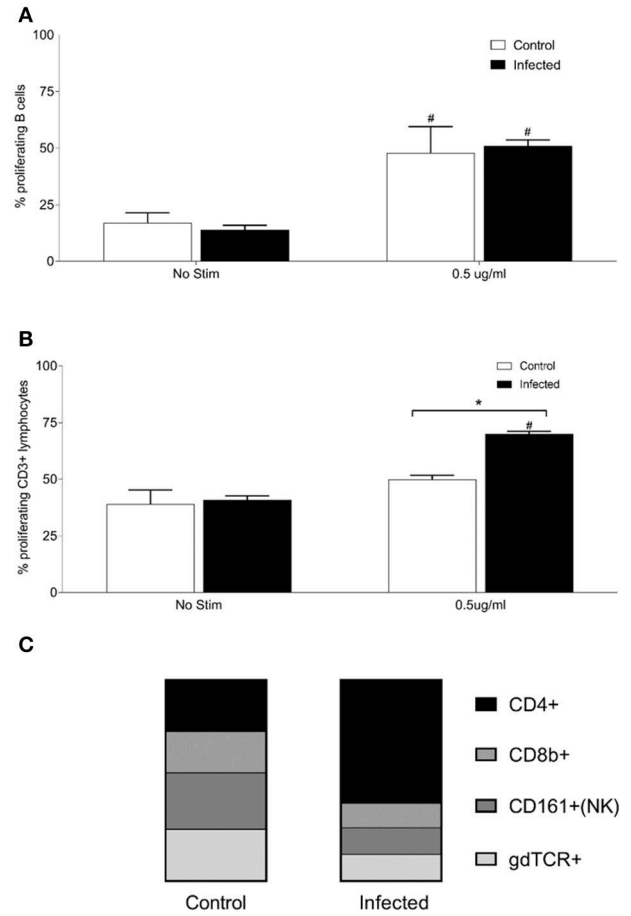


FIGURE 5 | Proliferative responses and cellular phenotypes of lymphocytes isolated from the renal lymph node of 15 infected and nine control rats. Lymphocytes were isolated from the RLN, pooled into groups of 3 animals, and stimulated with purified OM fraction from *Leptospira*. Percentage of proliferating B cells (CD3−, CD19+, or B220+) **(A)**, percentage of proliferating T cells (CD3+) **(B)** and phenotype by proportion of proliferating CD3+ cells stimulated with 0.5 μg/ml OM antigen **(C)**. *Indicates statistical significance between infected and control; #Indicates statistical significance between OM antigen stimulated wells and no stimulant (No Stim) wells within the same group.

Regardless, the use of an inbred immunocompetent rat model of persistent renal colonization, using different doses of inoculum at a range of time-points post-infection, provides for the further characterization of the host cellular immune response, in both systemic and local immune sites, to understand the pathophysiology of persistent renal colonization over time in an immunocompetent reservoir host of infection.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JN and JW-W. Performed the experiments: JN, JW-W, and RH. Contributed resources, reagents, materials, and analysis tools: JN, JW-W, RH, MP, and DA. Wrote the paper: JN. Revised the paper: JN, JW-W, RH, MP, and DA.

FUNDING

JN, JW-W, RH, MP, and DA are US Department of Agriculture employees. Work was completed in the course of their assigned duties in relation to project number 5030-32000-223-00-D.

ACKNOWLEDGMENTS

We thank Prof. Melissa Caimano, University of Connecticut Health Center, for kindly providing antisera specific for FlaA of *Treponema*, Ami Frank for immunohistochemistry and gene expression assays, and Sam Humphrey for flow cytometry. USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00066/full#supplementary-material

Supplementary Figure 1 | Gating strategy for flow cytometry. A representative example using lymphocytes purified from the spleen of experimentally infected rats is provided. Live cells were gated using Zombie Yellow Live/Dead discriminator dye **(A)**. Live cells were then gated on forward scatter (FSC) and side scatter (SSC) pattern consistent with proliferating lymphocytes **(B)**. Proliferating lymphocytes were gated on decrease in fluorescence of Cell Trace Violet cellular proliferation dye **(C)**. Dotted line is unstimulated cells, gray histogram is ConA 1 μg/ml stimulated cells and black is 0.5 μg/ml OM antigen stimulated cells. Proliferating cells were then gated into CD3+ and CD3– populations against an irrelevant marker **(D)**. Proliferating B cell population was defined in CD3– subset as being CD19+ and/or CD45R/B220+ **(E)**. Proliferating T cell subset (CD3+) was further phenotyped into CD4+ vs. CD8b+ **(F)**, gamma-delta TCR+ (γδTCR) **(G)** and NK (CD161a+) **(H)** subsets respectively.

Supplementary Table 1 | Antibodies used for flow cytometry.

Supplementary Table 2 | List of genes used in custom RT-PCR array.

REFERENCES

Agudelo-Flórez, P., Murillo, V. E., Londoño, A. F., and Rodas, J. D. (2013). Histopathological kidney alterations in rats naturally infected with *Leptospira*. *Biomedica* 33(Suppl. 1), 82–88. doi: 10.7705/biomedica.v33i0.686

Athanazio, D. A., Silva, E. F., Santos, C. S., Rocha, G. M., Vannier-Santos, M. A., McBride, A. J., et al. (2008). *Rattus norvegicus* as a model for persistent renal colonization by pathogenic *Leptospira interrogans*. *Acta Trop.* 105, 176–180. doi: 10.1016/j.actatropica.2007.10.012

Belkaid, Y., and Rouse, B. T. (2005). Natural regulatory T cells in infectious disease. *Nat. Immunol.* 6, 353–360. doi: 10.1038/ni1181

Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., et al. (2003). Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* 3, 757–771. doi: 10.1016/S1473-3099(03)00830-2

Bolin, C. A., and Alt, D. P. (2001). Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira borgpetersenii* serovar hardjo. *Am. J. Vet. Res.* 62, 995–1000. doi: 10.2460/ajvr.2001.62.995

Bonilla-Santiago, R., and Nally, J. E. (2011). Rat model of chronic leptospirosis. *Curr. Protoc. Microbiol.* Chapter 12:Unit12E.3. doi: 10.1002/9780471729259.mc12e03s20

Challa, S., Nally, J. E., Jones, C., and Sheoran, A. S. (2011). Passive immunization with *Leptospira* LPS-specific agglutinating but not non-agglutinating monoclonal antibodies protect guinea pigs from fatal pulmonary hemorrhages induced by serovar Copenhageni challenge. *Vaccine* 29, 4431–4434. doi: 10.1016/j.vaccine.2011.04.041

Chassin, C., Picardeau, M., Goujon, J. M., Bourhy, P., Quellard, N., Darche, S., et al. (2009). TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial pathogen, *Leptospira interrogans*. *J. Immunol.* 183, 2669–2677. doi: 10.4049/jimmunol.0900506

Cole, J. R., Sulzer, C. R., and Pursell, A. R. (1973). Improved microtechnique for the leptospiral microscopic agglutination test. *Appl. Microbiol.* 25, 976–980.

Costa, F., Ribeiro, G. S., Felzemburgh, R. D., Santos, N., Reis, R. B., Santos, A. C., et al. (2014). Influence of household rat infestation on *Leptospira* transmission in the urban slum environment. *PLoS Negl. Trop. Dis.* 8:e3338. doi: 10.1371/journal.pntd.0003338

Costa, F., Wunder, E. A. Jr., De Oliveira, D., Bisht, V., Rodrigues, G., Reis, M. G., et al. (2015). Patterns in leptospira shedding in norway rats (*Rattus norvegicus*) from Brazilian Slum Communities at high risk of disease transmission. *PLoS Negl. Trop. Dis.* 9:e0003819. doi: 10.1371/journal.pntd.0003819

Coutinho, M. L., Choy, H. A., Kelley, M. M., Matsunaga, J., Babbitt, J. T., Lewis, M. S., et al. (2011). A LigA three-domain region protects hamsters from lethal infection by *Leptospira interrogans*. *PLoS Negl. Trop. Dis.* 5:e1422. doi: 10.1371/journal.pntd.0001422

Ellis, W. A. (2015). Animal leptospirosis. *Curr. Top. Microbiol. Immunol.* 387, 99–137. doi: 10.1007/978-3-662-45059-8_6

Fitzgerald, T. J. (1992). The Th1/Th2-like switch in syphilitic infection: is it detrimental? *Infect. Immun.* 60, 3475–3479.

Gasteiger, G., and Rudensky, A. Y. (2014). Interactions between innate and adaptive lymphocytes. *Nat. Rev. Immunol.* 14, 631–639. doi: 10.1038/nri3726

Godfrey, D. I., Uldrich, A. P., McCluskey, J., Rossjohn, J., and Moody, D. B. (2015). The burgeoning family of unconventional T cells. *Nat. Immunol.* 16, 1114–1123. doi: 10.1038/ni.3298

Haake, D. A., and Levett, P. N. (2015). Leptospirosis in humans. *Curr. Top. Microbiol. Immunol.* 387, 65–97. doi: 10.1007/978-3-662-45059-8_5

Ido, Y., Hoki, R., Ito, H., and Wani, H. (1917). The rat as a carrier of *Spirocheta icterohaemorrhagiae*, the causative agent of Weil's disease (spirochaetosis icterohaemorrhagica. *J. Exp. Med.* 26, 341–353. doi: 10.1084/jem.26.3.341

Iwakura, Y., Nakae, S., Saijo, S., and Ishigame, H. (2008). The roles of IL-17A in inflammatory immune responses and host defense against pathogens. *Immunol. Rev.* 226:57–79. doi: 10.1111/j.1600-065X.2008.00699.x

Libonati, H., Pinto, P. S., and Lilenbaum, W. (2017). Seronegativity of bovines face to their own recovered leptospiral isolates. *Microb. Pathog.* 108, 101–103. doi: 10.1016/j.micpath.2017.05.001

Lu, L. L., Suscovich, T. J., Fortune, S. M., and Alter, G. (2018). Beyond binding: antibody effector functions in infectious diseases. *Nat. Rev. Immunol.* 18, 46–61. doi: 10.1038/nri.2017.106

Matsui, M., Rouleau, V., Bruyère-Ostells, L., and Goarant, C. (2011). Gene expression profiles of immune mediators and histopathological findings in animal models of leptospirosis: comparison between susceptible hamsters and resistant mice. *Infect. Immun.* 79, 4480–4492. doi: 10.1128/IAI.05727-11

McBride, A. J., Athanazio, D. A., Reis, M. G., and Ko, A. I. (2005). Leptospirosis. *Curr. Opin. Infect. Dis.* 18, 376–386. doi: 10.1097/01.qco.0000178824.05715.2c

Miller, D. A., Wilson, M. A., and Beran, G. W.(1991). Survey to estimate prevalence of *Leptospira interrogans* infection in mature cattle in the United States. *Am. J. Vet. Res.* 52, 1761–1765.

Miller, J. N. (1971). *Spirochetes in Body Fluids and Tissues*. Springfield, IL: Charles, C. THomas.

Minter, A., Diggle, P. J., Costa, F., Childs, J., Ko, A. I., and Begon, M. (2017). Evidence of multiple intraspecific transmission routes for *Leptospira* acquisition in Norway rats (*Rattus norvegicus*). *Epidemiol. Infect.* 145, 3438–3448. doi: 10.1017/S0950268817002539

Monahan, A. M., Callanan, J. J., and Nally, J. E. (2008). Proteomic analysis of *Leptospira interrogans* shed in urine of chronically infected hosts. *Infect. Immun.* 76, 4952–4958. doi: 10.1128/IAI.00511-08

Monahan, A. M., Callanan, J. J., and Nally, J. E. (2009). Review paper: Host-pathogen interactions in the kidney during chronic leptospirosis. *Vet. Pathol.* 46, 792–799. doi: 10.1354/vp.08-VP-0265-N-REV

Murphy, K., and Weaver, C. (2016). Janeway's *Immunobiology*. New York, NY: Garland Science.

Naiman, B. M., Blumerman, S., Alt, D., Bolin, C. A., Brown, R., Zuerner, R., et al. (2002). Evaluation of type 1 immune response in naïve and vaccinated animals following challenge with *Leptospira borgpetersenii* serovar Hardjo: involvement of WC1+ γδ and CD4 T cells. *Infect. Immun.* 70, 6147–6157. doi: 10.1128/IAI.70.11.6147-6157.2002

Nally, J. E., Artiushin, S., and Timoney, J. F. (2001). Molecular characterization of thermoinduced immunogenic proteins Qlp42 and Hsp15 of *Leptospira interrogans*. *Infect. Immun.* 69, 7616–7624. doi: 10.1128/IAI.69.12.7616-7624.2001

Nally, J. E., Chantranuwat, C., Wu, X. Y., Fishbein, M. C., Pereira, M. M., Da Silva, J. J., et al. (2004). Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in a guinea pig model of severe pulmonary leptospirosis. *Am. J. Pathol.* 164, 1115–1127. doi: 10.1016/S0002-9440(10)63198-7

Nally, J. E., Chow, E., Fishbein, M. C., Blanco, D. R., and Lovett, M. A. (2005). Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. *Infect. Immun.* 73, 3251–3260. doi: 10.1128/IAI.73.6.3251-3260.2005

Nally, J. E., Grassmann, A. A., Planchon, S., Sergeant, K., Renaut, J., Seshu, J., et al. (2017). Pathogenic leptospires modulate protein expression and post-translational modifications in response to mammalian host signals. *Front. Cell. Infect. Microbiol.* 7:362. doi: 10.3389/fcimb.2017.00362

Nally, J. E., Monahan, A. M., Miller, I. S., Bonilla-Santiago, R., Souda, P., and Whitelegge, J. P. (2011). Comparative proteomic analysis of differentially expressed proteins in the urine of reservoir hosts of leptospirosis. *PLoS ONE* 6:e26046. doi: 10.1371/journal.pone.0026046

Nally, J. E., Mullen, W., Callanan, J. J., Mischak, H., and Albalat, A. (2015). Detection of urinary biomarkers in reservoir hosts of leptospirosis by capillary electrophoresis-mass spectrometry. *Proteomics Clin. Appl.* 9, 543–551. doi: 10.1002/prca.201400205

Nally, J. E., Whitelegge, J. P., Bassilian, S., Blanco, D. R., and Lovett, M. A. (2007). Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. *Infect. Immun.* 75, 766–773. doi: 10.1128/IAI.00741-06

Raffray, L., Giry, C., Thirapathi, Y., Binois, F., Moiton, M. P., Lagrange-Xelot, M., et al. (2015). High leptospiremia is associated with low gamma-delta T cell counts. *Microbes Infect.* 17, 451–455. doi: 10.1016/j.micinf.2015.04.001

Richer, L., Potula, H. H., Melo, R., Vieira, A., and Gomes-Solecki, M. (2015). Mouse model for sublethal *Leptospira interrogans* infection. *Infect. Immun.* 83, 4693–4700. doi: 10.1128/IAI.01115-15

Rojas, P., Monahan, A. M., Schuller, S., Miller, I. S., Markey, B. K., and Nally, J. E. (2010). Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 1305–1309. doi: 10.1007/s10096-010-0991-2

Schuller, S., Sergeant, K., Renaut, J., Callanan, J. J., Scaife, C., and Nally, J. E. (2015). Comparative proteomic analysis of lung tissue from guinea pigs with Leptospiral Pulmonary Haemorrhage Syndrome (LPHS) reveals a decrease in abundance of host proteins involved in cytoskeletal and cellular organization. *J. Proteomics* 122, 55–72. doi: 10.1016/j.jprot.2015.03.021

Sterling, C. R., and Thiermann, A. B. (1981). Urban rats as chronic carriers of leptospirosis: an ultrastructural investigation. *Vet. Pathol.* 18, 628–637. doi: 10.1177/030098588101800508

Thiermann, A. B. (1981). The Norway rat as a selective chronic carrier of *Leptospira icterohaemorrhagiae*. *J. Wildl. Dis.* 17, 39–43. doi: 10.7589/0090-3558-17.1.39

Torgerson, P. R., Hagan, J. E., Costa, F., Calcagno, J., Kane, M., Martinez-Silveira, M. S., et al. (2015). Global burden of leptospirosis: estimated in terms of disability adjusted life years. *PLoS Negl. Trop. Dis.* 9:e0004122. doi: 10.1371/journal.pntd.0004122

Tucunduva de Faria, M., Athanazio, D. A., Gonçalves Ramos, E. A., Silva, E. F., Reis, M. G., and Ko, A. I. (2007). Morphological alterations in the kidney of

rats with natural and experimental Leptospira infection. *J. Comp. Pathol.* 137, 231–238. doi: 10.1016/j.jcpa.2007.08.001

Volz, M. S., Moos, V., Allers, K., Luge, E., Mayer-Scholl, A., Nöckler, K., et al. (2015). Specific CD4+ T-Cell reactivity and cytokine release in different clinical presentations of leptospirosis. *Clin. Vaccine Immunol.* 22, 1276–1284. doi: 10.1128/CVI.00397-15

Witchell, T. D., Eshghi, A., Nally, J. E., Hof, R., Boulanger, M. J., Wunder, E. A. Jr., et al. (2014). Post-translational modification of LipL32 during *Leptospira interrogans* infection. *PLoS Negl. Trop. Dis.* 8:e3280. doi: 10.1371/journal.pntd.0003280

Wunder, E. A. Jr., Figueira, C. P., Santos, G. R., Lourdault, K., Matthias, M. A., Vinetz, J. M., et al. (2016). Real-Time PCR reveals rapid dissemination of *Leptospira interrogans* after intraperitoneal and conjunctival inoculation of hamsters. *Infect. Immun.* 84, 2105–2115. doi: 10.1128/IAI.00094-16

Zuerner, R. L., Alt, D. P., Palmer, M. V., Thacker, T. C., and Olsen, S. C. (2011). A *Leptospira borgpetersenii* serovar Hardjo vaccine induces a Th1 response, activates NK cells, and reduces renal colonization. *Clin. Vaccine Immunol.* 18, 684–691. doi: 10.1128/CVI.00288-10

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 19 June 2018
doi: 10.3389/fcimb.2018.00193

Genomic Comparison Among Global Isolates of *L. interrogans* Serovars Copenhageni and Icterohaemorrhagiae Identified Natural Genetic Variation Caused by an Indel

Luciane A. Santos[1,2†], Haritha Adhikarla[1†], Xiting Yan[3], Zheng Wang[3], Derrick E. Fouts[4], Joseph M. Vinetz[5], Luiz C. J. Alcantara[2], Rudy A. Hartskeerl[6], Marga G. A. Goris[6], Mathieu Picardeau[7], Mitermayer G. Reis[2], Jeffrey P. Townsend[3], Hongyu Zhao[3], Albert I. Ko[1,2] and Elsio A. Wunder Jr.[1,2*]*

[1] Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, United States, [2] Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Brazil, [3] Department of Biostatistics, Yale School of Public Health, New Haven, CT, United States, [4] J. Craig Venter Institute, Rockville, MD, United States, [5] Division of Infectious Diseases, Department of Medicine, University of California San Diego School of Medicine, La Jolla, CA, United States, [6] Royal Tropical Institute, KIT Biomedical Research, Amsterdam, Netherlands, [7] Biology of Spirochetes Unit, Institut Pasteur, Paris, France

OPEN ACCESS

Edited by:
Guoquan Zhang, University of Missouri, United States

Reviewed by:
Nuvee Prapasarakul, Chulalongkorn University, Thailand
Sérgio Jorge, Universidade Federal de Pelotas, Brazil

***Correspondence:**
Albert I. Ko
albert.ko@yale.edu
Elsio A. Wunder Jr.
elsio.wunder@yale.edu

[†]These authors have contributed equally to this work.

Received: *16 January 2018*
Accepted: *22 May 2018*
Published: *19 June 2018*

Citation:
Santos LA, Adhikarla H, Yan X, Wang Z, Fouts DE, Vinetz JM, Alcantara LCJ, Hartskeerl RA, Goris MGA, Picardeau M, Reis MG, Townsend JP, Zhao H, Ko AI and Wunder EA Jr (2018) Genomic Comparison Among Global Isolates of L. interrogans Serovars Copenhageni and Icterohaemorrhagiae Identified Natural Genetic Variation Caused by an Indel. Front. Cell. Infect. Microbiol. 8:193. doi: 10.3389/fcimb.2018.00193

Leptospirosis is a worldwide zoonosis, responsible for more than 1 million cases and 60,000 deaths every year. Among the 13 pathogenic species of the genus *Leptospira*, serovars belonging to *L. interrogans* serogroup Icterohaemorrhagiae are considered to be the most virulent strains, and responsible for majority of the reported severe cases. Serovars Copenhageni and Icterohaemorrhagiae are major representatives of this serogroup and despite their public health relevance, little is known regarding the genetic differences between these two serovars. In this study, we analyzed the genome sequences of 67 isolates belonging to *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae to investigate the influence of spatial and temporal variations on DNA sequence diversity. Out of the 1072 SNPs identified, 276 were in non-coding regions and 796 in coding regions. Indel analyses identified 258 indels, out of which 191 were found in coding regions and 67 in non-coding regions. Our phylogenetic analyses based on SNP dataset revealed that both serovars are closely related but showed distinct spatial clustering. However, likelihood ratio test of the indel data statistically confirmed the presence of a frameshift mutation within a homopolymeric tract of *lic12008* gene (related to LPS biosynthesis) in all the *L. interrogans* serovar Icterohaemorrhagiae strains but not in the Copenhageni strains. Therefore, this internal indel identified can genetically distinguish *L. interrogans* serovar Copenhageni from serovar Icterohaemorrhagiae with high discriminatory power. To our knowledge, this is the first study to identify global sequence variations (SNPs and Indels) in *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae.

Keywords: *Leptospira*, leptospirosis, Copenhageni, Icterohaemorrhagiae, whole-genome sequencing, SNPs, Indels, phylogeny

BACKGROUND

Leptospirosis is a zoonosis with worldwide distribution and considered endemic in developing countries and tropical regions. This life-threatening disease is caused by pathogenic spirochetes from the genus *Leptospira* (Bharti et al., 2003; Mcbride et al., 2005; Ko et al., 2009). Globally, leptospirosis is conservatively estimated to cause 1.03 million cases and 58,900 deaths each year (Costa et al., 2015). Transmission of leptospirosis requires continuous enzootic circulation of the pathogen among animal reservoirs. This zoonosis is maintained in nature through chronic renal infection of carrier animals, with rodents and other small mammals being the most important reservoirs. Humans and other accidental hosts get infected by direct contact with infected animals or with contaminated water or soil (Bharti et al., 2003; Ko et al., 2009).

Pathogenic *Leptospira* includes 13 species and have been classified into over 250 distinct serotypes (Reis et al., 2008; Lehmann et al., 2014; Picardeau, 2017). In humans, severe leptospirosis is frequently associated with *L. interrogans* serogroup Icterohaemorrhagiae (Bharti et al., 2003). Currently majority of the human cases caused by Icterohaemorrhagiae serogroup are attributed to serovar Copenhageni strains. Although *Rattus norvegicus* is recognized as the main reservoir for serovar Copenhageni, rodents of the *Rattus* spp. are considered as the reservoir for both serovars (Ido et al., 1917; Vinetz, 1996; Ko et al., 1999; de Faria et al., 2008). Furthermore, there is no apparent difference in terms of disease outcome between *L. interrogans* Copenhageni and Icterohaemorrhagiae serovars. In spite of being serologically distinct in serotyping methods, these serovars are still considered to be genetically similar (Kmety and Dikken, 1993; Majed et al., 2005). Therefore researchers are trying to understand the phenotypical and genotypic differences between these serovars with little success.

Genotyping methods such as multilocus variable-number tandem repeat (VNTR), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) employed to distinguish *L. interrogans* Copenhageni and Icterohaemorrhagiae serovars lack enough discriminatory power (Barocchi et al., 2001; Bharti et al., 2003; Majed et al., 2005; Galloway and Levett, 2010; Romero et al., 2011). Multispacer sequence typing (MST) represents an evolved genotyping method for the serovar identification of *L. interrogans* serogroup Icterohaemorrhagiae (Zilber et al., 2014). Though serovar variation has been related to lipopolysaccharide (LPS) structure, specifically involving the biosynthesis locus (rfb cluster) of the O-antigen, none of the above molecular typing methods are directly related to this cluster (Faine, 1994; Llanes et al., 2016). Further comparative genomic analysis of *L. interrogans* serovars Icterohaemorrhagiae and Copenhageni may reveal the differences essential for development of novel molecular serotyping techniques (Moreno et al., 2016).

DNA polymorphisms such as single nucleotide polymorphisms (SNPs), insertions and deletions (Indels), and other larger rearrangements were successfully employed to study sequence diversity among closely related but distinct populations (Gutacker et al., 2002; Joshi et al., 2012). The use of next generation sequencing (NGS) data to detect DNA polymorphisms in the context of whole-genome analysis has been previously reported in pathogenic bacteria like *Salmonella typhi*, *Brucella* spp. and *Bacillus anthracis* (Fournier et al., 2014). Therefore, whole-genome sequencing might serve as a robust and unbiased method to resolve intraspecies relationships in *Leptospira*.

Genome-wide identification of SNPs and Indels in *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae would advance our understanding of genomic diversity of these strains isolated from various geographic locations and their evolution. Identifying the genotypic differences between both serovars can improve our understanding of their evolutionary relationships in diverse epidemiological settings. This study of genomic variations will also facilitate the development of new molecular markers to differentiate pathogenic serovars and will further aid in the leptospirosis prevention strategies. Hence we performed whole-genome sequencing of 67 different strains of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae and conducted genome-wide analyses to identify serovar-specific differences.

MATERIALS AND METHODS

Leptospira Isolates

A total of 67 strains of *L. interrogans* serogroup Icterohaemorrhagiae, including 55 serovar Copenhageni isolates and 12 serovar Icterohaemorrhagiae isolates, were included in this study. These strains were isolated from different geographic locations and hosts, and the years of isolation ranged from 1915 to 2012 (**Table S1**). To include more strains for validation, eight *L. interrogans* serovar Icterohaemorrhagiae and seven serovar Copenhageni strains, whose genomes were not sequenced, were also included in this study for Sanger sequencing and/or function analyses (**Table S1**).

Culture, Genomic DNA Extraction, and Sequencing

The *Leptospira* strains were cultured in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) (Johnson and Harris, 1967) media incubated at 29°C with moderate shaking at 100 rpm. DNA was extracted from late-log phase cultures using the Maxwell 16 cell DNA purification kit along with the Maxwell DNA extraction system (Promega). The quality and concentration of DNA was measured by spectrophotometry using the NanoDrop system (Thermo Scientific, DE, USA) and by fluorometic assay using the Quanti-iT PicoGreen dsDNA assay kit (Invitrogen).

Genomes were sequenced for all 67 strains described in this study and corresponding datasets were recruited for further analyses. Genomic sequencing was done at the J. Craig Venter Institute (JCVI) using an Illumina/Solexa Genome Analyzer II, and at the Yale Center for Genome Analysis (YCGA) using the Illumina HiSeq 2000. Whole genome reads for each isolate were deposited at NCBI in the Sequence Read Archive (SRA) database (accession numbers in **Table S1**).

Serological Characterization of Isolates

The microscopic agglutination test (MAT) was used to type *Leptospira* isolates. For serogrouping, a standard 19 panel rabbit polyclonal antisera against reference serovars representing 12 different serogroups was used, as previously described (Reis et al., 2008). For serotyping, we used different monoclonal antibodies (F89 C12-6, F70 C14, F70 C24-20, and F12 C3-11 - KIT-Biomedical Research, Amsterdam) to classify isolates of serogroup Icterohaemorrhagiae as serovar Copenhageni or serovar Icterohaemorrhagiae.

Sequence Analysis Pipeline

Reads were mapped to the *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni L1-130 strain reference genome (Nascimento et al., 2004) (Accession numbers: NC005823 and NC005824) using Stampy tool (Lunter and Goodson, 2011). The replicated alignment removal and local realignment were done using Samtools (Li et al., 2009). The processed mapping results were further analyzed for SNP calling using Samtools. Called SNPs were filtered to have a quality score cut-off >30. CLC Genomics workbench (CLC Genomics 6.0.4) was used to call Indels, and those with coverage lower than 5x were filtered. The Samtools pipeline exhibited better rates of consistency for SNPs calling while CLC was consistent for Indel calling (**Figure 1**, **Tables S2**, **S3**). In our reference-guided approach, we subsequently analyzed the un-mapped reads and did not detect any plasmids or misplaced contigs. To evaluate the presence of complex mutations (heterozygosis), Sanger sequencing and RFLP were performed for 3 regions. All analyses showed that the alternative allele was not present indicating a sequence error in some of the reads. Based on these analyses all the complex SNPs were excluded from this pipeline.

Phylogenetic Analyses

Phylogenetic analyses of SNPs across the whole-genome were used to infer the relationships among the 67 isolates of *L. interrogans* serogroup Icterohaemorrhagiae collected from diverse geographical locations. The reference strain *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni L1-130 (Nascimento et al., 2004) was included in the phylogenetic analysis. *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai (Ren et al., 2003) was used as an outgroup. The length of the sequence alignment consisted of 1,731 variable sites, out of which 1072 variations were detected in this study from the sequences of serovars Copenhageni and Icterohaemorrhagiae. Additional 659 variable sites were included from the outgroup sequence serovar Lai. The Maximum Likelihood (ML) phylogeny was inferred using PAUP* (Rogers and Swofford, 1998) applying the GTR model of nucleotide substitution and gamma shape parameter. Bootstrap analysis (1,000 replicates) was used to calculate the

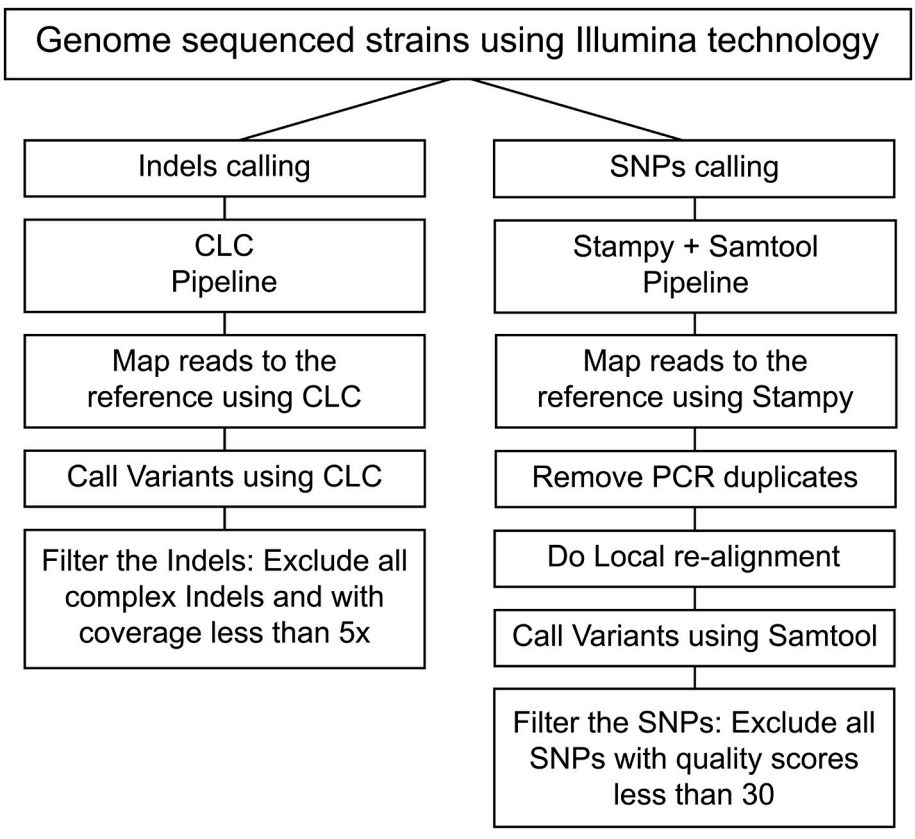


FIGURE 1 | Pipeline process to identify SNPs and Indels. The flowchart represents the pipelines employed for calling SNPs (Stampy and Samtools) and Indels (CLC).

statistical support of the tree branches. Bayesian trees were also inferred including the years and country of isolation in the tree construction parameters using BEAST v.1.8 package (Drummond and Rambaut, 2007). For this dataset, we only included the 57 sequences that presented complete data from year and country of isolation (**Table S1**). The models tested were the strict molecular clock with constant population size prior and the relaxed molecular clock using the constant population size, Bayesian Skyline Plot (BSP), Skyride and exponential growth priors. The parameters for each model were estimated using the Monte Carlo Markov Chain (MCMC) method (50,000,000 generations with sampling every 5,000 generations). The tested models were compared calculating the Bayes Factor (BF). Using TreeAnnotator v1.4.8, included in the BEAST package (Drummond and Rambaut, 2007), the maximum clade credibility tree was summarized from the posterior tree distribution after a 50% burn-in, for each dataset (Drummond and Rambaut, 2007). MacClade was used to identify the frequency of unambiguous changes among various countries from where isolates originated in the tree (Maddison and Maddison, 1989).

Statistical Analyses

Genotypes of *L. interrogans* serogroup Icterohaemorrhagiae serovars Icterohaemorrhagiae and Copenhageni strains were compared based on the log likelihood ratio test. For a given SNP or indel, two binomial distributions were fitted for the number of alternative alleles observed separately in Icterohaemorrhagiae and Copenhageni strains by maximizing the likelihood. The supposed maximized likelihood of Icterohaemorrhagiae and Copenhageni were denoted as L_I and L_C. Then another binomial distribution was fitted for the number of alternative allele by treating all strains from Icterohaemorrhagiae and Copenhageni as one group. If the maximized likelihood of this binomial distribution is $L_{I\ \&C}$, then the log likelihood ratio is calculated as $-2\log(\frac{L_{I\ \&C}}{(L_I \cdot L_C)})$. The *P* value was then calculated by comparing this observed log likelihood ratio to the Chi-squared distribution with 1 degree of freedom. To detect the degree of clustering of the genetic variation, a Principal Component Analysis (PCA) was performed using the SNPs. These analyses were performed using R.

Identification of Internal Indel in *L. interrogans* Serovars Icterohaemorrhagiae

Sanger sequencing confirmed the observed *lic12008* mutation using gene specific primers (forward 5′TAGGTTGGCACG AAGGTTCT3′ and reverse 5′TTTTTCCGGGAACTCCAAC3′) Sequencher 5.2 (Sequencher® Gene Codes Corporation) was used to conduct the sequence analysis, and new sequences were aligned with the reference strain to identify the presence of the mutation. A total of 13 strains belonging to *L. interrogans* serovar Icterohaemorrhagiae (WGS for 5 strains) and 9 strains (WGS for 2 strains) of serovar Copenhageni were also analyzed (**Table S1**). BLAST analyses identified homologous sequences of LIC12008 at nucleotide and protein levels. Domain analysis of LIC12008 protein was performed using NCBI CD-search and Pfam 27.0 sequence search tools (Finn et al., 2008).

Isolation of RNA and Quantitative Reverse Transcription PCR (RT-qPCR)

L. interrogans serovar Icterohaemorrhagiae strains 201000458, 201000456 and serovar Copenhageni strain L1-130 were cultured to a density of 10^8 bacteria per mL at 29°C with shaking. Cultures were harvested via centrifugation at 3,200 *g* and RNA was extracted for two biological replicates using the TRIzol (Invitrogen) method, as previously described. Ambion® TURBO DNA-free™ DNase Treatment kit was employed to remove contaminating DNA from RNA preparations. The concentration of RNA was determined using a Spectrophotometer (NanoDrop). The high capacity cDNA reverse transcription kit (Life Technologies) was employed for conversion of total RNA to single-stranded cDNA. Two primer sets were used to assess the impact of frameshift mutation on expression of LIC12008 (**Table S7**). First primer set (12008 T1) amplified a fragment of 126 bp in region encompassing nucleotides from 29 to 155 bp before the frameshift mutation. A second primer set (12008 T2) amplified a fragment of 133 bp after the mutation (from nucleotide 335–468) (**Table S7**).

The qPCR was carried out on 7500 fast real-time PCR (ABI, USA) using iQ™ SYBR® Green supermix (Biorad) according to manufacturer's instructions. The thermal cycling conditions used in the qPCR were 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. The specificity of the SYBR green PCR signal was confirmed by melt curve analysis. In RT-qPCR experiments, *flaB* gene was used as an endogenous control and reference strain employed was *L. interrogans* serovar Copenhageni strain L1-130. Relative quantification analysis was performed using the comparative C_t method, and relative gene expression was calculated by using the $2^{-\Delta\Delta}$Ct method (Schmittgen and Livak, 2008).

In Vivo Characterization

To test whether the *lic12008* mutation impacts virulence, *in vivo* experiments were performed in the hamster model of infection. Two strains of *L. interrogans* serovar Icterohaemorrhagiae (201000458, 201000456) were tested and compared to *L. interrogans* serovar Copenhageni strain L1-130. Groups of three 21 day old male Golden Syrian hamsters were infected with doses of 10^2 and 10^8 leptospires by intraperitoneal (IP) and conjunctival routes as described previously (Wunder et al., 2016).

Animals were monitored twice daily for endpoints including signs of disease and death up to 21-days post-infection. Surviving animals 21-days after infection or moribund animals at any time presenting with difficulty moving, breathing or signs of bleeding or seizure were sacrificed by CO_2 inhalation (Wunder et al., 2016).

Ethics Statement

Animal protocols and work were approved and conducted under the guidelines of the Yale Institutional Animal Care and Use Committee (IACUC), under protocol #2017–11424. The Yale

IACUC strictly adheres to all Federal and State regulations, including the Animal Welfare Act, those specified by Public Health Service, and the US Department of Agriculture and uses the *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* as a guide for animal studies.

RESULTS AND DISCUSSION

Pipeline for Whole-Genome Mapping

In this study, a total of 67 strains were sequenced, out of which 55 strains belonged to *L. interrogans* serovar Copenhageni and 12 strains belonged to *L. interrogans* serovar Icterohaemorrhagiae (**Table S1**). Greater number of *L. interrogans* serovar Copenhageni sequences were included in this study as this serovar is more prevalent than serovar Icterohaemorrhagiae and has greater number of isolates. All the 67 strains that were included in this study were confirmed by MAT using polyclonal sera for confirmation of Icterohaemorrhagiae serogroup (serogrouping) and monoclonal sera for differentiating Icterohaemorrhagiae and Copenhageni serovars. Serogrouping and serotyping by MAT (data not shown) confirmed the relatedness of the isolates to serogroup Icterohaemorrhagiae, and their identity to serovars Copenhageni or Icterohaemorrhagiae (**Table S1**). Stampy and Samtools were used for read mapping and SNP identification, respectively. Indels for both mapping and identification were analyzed using the CLC genome workbench. The pipeline used for identification of SNPs and indels was validated in this study by re-sequencing seven *Leptospira* isolates. Sequences from each of the seven isolates were analyzed and the selection of the best pipeline was made based on identification of the highest overlap percentage of SNPs and/or indels found in both sequences (**Figure 1**, **Tables S2**, **S3**). The information related to reads and assembly quality for these sequenced genomes can be found in **Table S4**.

L. interrogans serovar Copenhageni strain Fiocruz L1-130, sequenced using shotgun technology, was used as the reference genome (Accession numbers: NC005823 and NC005824) (Nascimento et al., 2004). Comparison of genome of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 strain re-sequenced using Illumina technology with the previously published genome sequence resulted in identification of 66 SNPs and 62 indels. Of these, 45 SNPs and 46 indels had a distribution frequency of 97% or higher in all the 132 Copenhageni strains sequenced by our group (55 included in this study and 77 from a different study) (data not shown). This higher distribution frequency of SNPs and INDELs in all the above strains might be attributed to the propagation of sequencing errors from the reference *L. interrogans* Fiocruz L1-130 sequence. In order to avoid those mutations with high frequency from the reference *L. interrogans* Fiocruz L1-130 sequence we excluded them from our analysis.

For this manuscript, we used a reference-guided approach to identify the basic differences between the genomes. However, subsequently we also employed *de novo* assembly but it did not improve the outcome. Furthermore, we checked the unmapped reads and performed a de novo assembly with those reads but this approach also did not find any misplaced contigs or reads. In our data, the assembly covered from 97.4 to 99.99% of the genome.

Characteristics of the Mutations Detected in *L. interrogans* Serogroup Icterohaemorrhagiae Serovars Copenhageni and Icterohaemorrhagiae Strains

Whole genome sequencing enabled us to study the genome-wide variations of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae. Cumulatively, we identified 1,072 SNPs in 67 isolates, of which 276 were in non-coding region and 796 in coding regions (**Table 1** and **Table S5**). SNPs in coding regions were distributed in 594 different genes, and 115 of those had two or more SNPs in the same gene (**Table 1** and **Table S5**). Of the identified mutations in coding regions, 258 were synonymous and 538 were non-synonymous. The frequency of distribution of SNPs indicated a high proportion of non-synonymous to synonymous changes, at a ratio of 2:1. Previous comparative studies of closely related *B. anthracis*, *M. bovis,* and *Chlamydia* strains also revealed the high frequency of non-synonymous SNPs (Jordan et al., 2002; Read et al., 2002; Garnier et al., 2003). Therefore, a high proportion of non-synonymous mutations in serovars Copenhageni or Icterohaemorrhagiae could be due to their recent emergence suggesting that purifying selection might have had insufficient time to remove these slightly deleterious mutations as observed in other pathogenic bacteria (Harrison, 2013). Alternatively, this high proportion of non-synonymous mutations in both the serovars could also be an indication that some of these genes might be under positive selection.

We also identified 258 indels, of which 191 (107 deletions and 84 insertions) were found in coding regions and 67 (26 deletions and 41 insertions) in non-coding regions. Indels in coding-region were distributed in 153 different genes with 26 genes harboring two or more mutations (**Table 1** and **Table S6**). Of the 191 indels identified in coding region 183 indels caused a frameshift in the reading frames. The resulting amino acid changes and other characteristics have been represented in **Table S6**. However more functional studies are required to understand if these affected genes are related to any phenotypic consequences.

TABLE 1 | Classification and total number of identified mutations among isolates of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae.

Mutation	Classification	Coding region	Non-coding region	Total
SNPs	Synonymous	258	NA	258
	Non-synonymous	538	NA	538
	Total	796	276	1,072
Indels	Insertion	84	41	125
	Deletion	107	26	133
	Total	191	67	258

NA, Not applied.

Our results further identified genes with higher frequency of SNPs and indels (**Table 2**). However, we could not correlate this high frequency of compound mutations (multiple mutations on the same gene) with any of the phenotypic outcomes. It is possible that some of these multiple indels in the same gene could serve as compensatory indels thereby restoring the translation frame and making it a less deleterious mutation (Liu et al., 2015). Given the caveat that most of the genes in the *Leptospira* genome are annotated as hypothetical proteins or have less homology to the characterized proteins, it might be difficult to phenotypically evaluate the compensatory indels.

To our knowledge, this is the first large-scale study to identify global sequence variations among *L. interrogans* Copenhageni and Icterohaemorrhagiae serovars.

Phylogenetic Analysis

We constructed phylogenetic trees to gain insights into the spatial and temporal diversity of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae (**Figure 2** and **Figure S1**). The analysis was performed with all 67 strains isolated from different geographic locations, hosts and collected during a broad range of time (**Table S1**). A maximum likelihood (ML) tree was constructed based on the SNPs identified in each of the 67 strains, using *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai (Ren et al., 2003) as an outgroup (**Figure 2**). ML tree presented a topology where both serovars Copenhageni and Icterohaemorrhagiae clustered together with statistic support (**Figure 2** cluster 6). In order to better interpret the internal branches and relation between genomes in **Figure 2** we also constructed a Bayesian phylogenetic tree (**Figure S1**) without the outgroup serovar Lai. In both the trees (**Figure 2** and **Figure S1**) we observed that Icterohaemorrhagiae and Copenhageni genomes clustered together with statistical support (represented by bootstrap for the ML tree and posterior probability for the Bayesian analyses). Therefore, our phylogenetic analyses indicated a genetic relatedness of the *L. interrogans* Copenhageni and Icterohaemorrhagiae serovars.

Despite phylogenetic relatedness, clones of strains from the Icterohaemorrhagiae serogroup seem to have evolved and adapted in different locations. Phylogenetic analysis showed seven geographic clades that corresponded spatially with the potential origins of the isolates (**Table 3**, **Figure 2**). The bootstrap support of higher than 70% was employed based on the general consensus (Hillis and Bull, 1993) and statistical support for these clades was further confirmed by principal components analysis (PCA) (data not shown). These clades include isolates from Brazil (clade 1A to 1F), Hawaii (USA, clade 2), French Polynesia (clade 3), Egypt, Denmark, Netherlands (clade 4), Japan (clade 5), Guadalupe and Slovenia (clade 6) and Colombia (clade 7) (**Table 3**). To substantiate our results for geographical clustering, Bayesian analysis was performed for 57 isolates of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae, which had specific information related to their location and year of isolation. The temporal and spatial information was incorporated into this analysis and a relaxed clock with Bayesian skyline plot was selected as the best explanatory model. The resulting tree for spatiotemporal analyses was similar to the ML tree, with distinct geographic clades from Brazil, Colombia, Guadalupe, French Polynesia, Hawaii, Egypt, and Japan (**Figure S1**).

Isolates of *L. interrogans* serovar Copenhageni from Salvador, Brazil, were significantly represented in both phylogenies and exhibited a mixed clustering pattern with isolates from other countries (**Figure 2**). The frequency of unambiguous changes between states over the maximum parsimonious trees (MPTs) was estimated in MacClade (**Figure S2**). Significant distribution of *L. interrogans* serovars was observed between European Union to Egypt (25%) and Guadeloupe to Slovenia (12.5%). Other small distribution events observed were from Brazil to Guyana and French Polynesia (1.5%). One of our important interpretations arising from the MacClade analysis was that strains from Egypt could have ancestral origins in Europe (**Figure S2**). Taken together, the MacClade analyses indicated few instances of distribution events within *L. interrogans* serovars across the globe and particularly in Salvador, Brazil.

Human connectivity with infectious rodent host reservoirs (*R. novergicus*) via transcontinental trading by mercantile ships from Europe, Africa and other continents is one plausible explanation for the introduction of various strains of *L. interrogans* serovar Copenhageni strains into Salvador. A recent study explored the global population structure of *R. novergicus* and suggested that brown rats expanded across Asia, Europe and North America through human settlements associated with Silk Road trade routes (Puckett et al., 2016). Therefore, it is possible that strains of *L. interrogans* serogroup Icterohaemorrhagiae might have been spread via rodent host reservoir. However, high level of sequence similarity among serogroup Icterohaemorrhagiae isolates indicates that specific SNPs/Indels observed were essential for their survival and prevalence in diverse habitats.

TABLE 2 | Identification of genes showing multiple SNPs and Indels in *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae.

Mutation	Gene ID	Gene Product	nV[a]	Chr[b]	Gene Position[c]	Gene Size (bp)
SNPs	LIC12896	Hypothetical protein	17	1	3498112	9,423
	LIC10502	Cytoplasmic protein	8	1	590325	8,364
Indels	LIC10900	Adenylate/guanylate	6	1	1085410	1,404
	LIC10674	Hypothetical protein	4	1	819364	432
	LIC13017	Acriflavin resistance	4	1	3673377	3,312

[a] *Total number of variants in corresponding gene.*
[b] *Chromosome.*
[c] *Gene position based on the L. interrogans serovar Copenhageni strain Fiocruz L1-130 reference genome (Accession numbers: NC005823 and NC005824).*

Identification of a Unique Indel in Serovar Icterohaemorrhagiae Strains

To identify the genetic mutation that could differentiate both serovars, a likelihood ratio test (LRT) of the SNP analyses was performed. LRT suggests that no SNPs distinguished these two

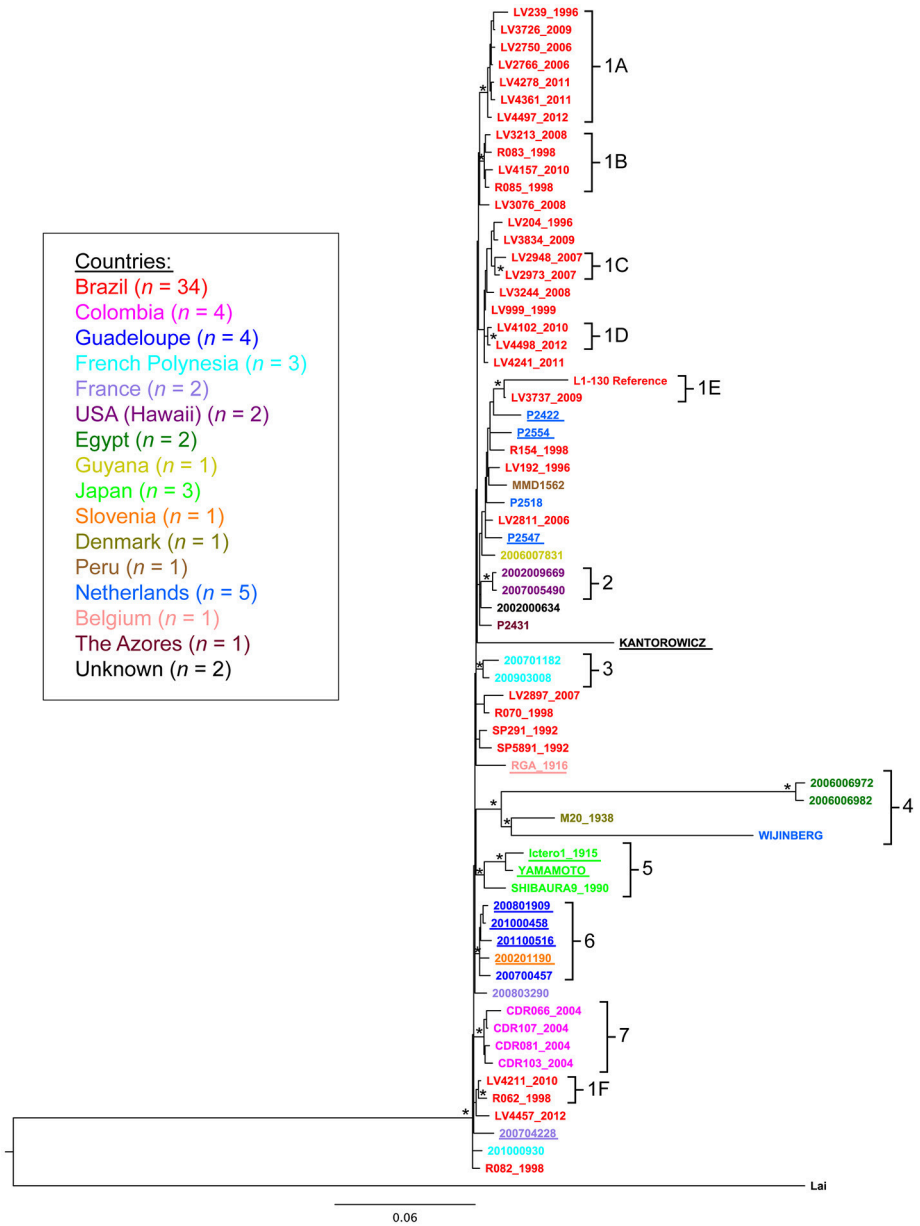


FIGURE 2 | Phylogenetic relationship among *Leptospira interrogans* serovar Copenhageni and Icterohaemorrhagiae strains. Maximum likelihood tree depicting phylogenetic relationship among *Leptospira interrogans* serovars Copenhageni and Icterohaemorrhagiae from different geographical locations. Phylogeny constructed using the SNPs sites. Asterisk (*) represents clusters with statistical Bootstrap support higher than 70%. The sequences from serovar Icterohaemorrhagiae are underlined. *L. interrogans* serovar Lai was used as an outgroup for this analysis. The strain sequences are colored by country of isolation following the legend. The clusters numbers are related to country of isolation: 1A–1F (Brazil), 2 (Hawaii, US), 3 (French Polynesia), 4 (Egypt, Netherlands and Denmark), 5 (Japan), 6 (Guadalupe and Slovenia), and 7 (Colombia).

serovars of **Icterohaemorrhagiae and Copenhageni**. However, LRT of the indel data was statistically consistent ($p = 0.039$) with the presence of a single base insertion of a thymine nucleotide within a polyT tract (9 bp long) in gene *lic12008* (**Figures 3A,B**) in all the *L. interrogans* serovar Icterohaemorrhagiae isolates. As an orthogonal approach, we ran parsimony informative test using our SNPs and it yielded 30% of parsimony-informative sites (data not shown). However, except for the one indel in *lic12008* locus we did not identify any statistically significant SNP's in our analyses.

The gene *lic12008* (726 bp) is located at nucleotide positions 2416786 to 2417511 of Chromosome 1 (reading frame of −2) in *L. interrogans* serovar Copenhageni Fiocruz L1-130 genome and was not found to be part of any operon. Sanger sequencing confirmed the presence of this insertion at the 5′ end of *lic12008* gene in all *L. interrogans* Icterohaemorrhagiae strains but its

absence in Copenhageni strains (data not shown). The identified insertion in *lic12008* gene resulted in a frameshift mutation at N terminal region (45th amino acid) of the corresponding protein (**Figure 3C**). This highly conserved change at amino acid level within the serovar Icterohaemorrhagiae might have evolutionary implications leading to its divergence from the Copenhageni serovar. A significant overrepresentation of frameshifts at N terminal region of proteins was also previously observed in pathogenic *P. aeruginosa* (Harrison, 2013).

TABLE 3 | Correlation of isolates based on the geographical clades from phylogenetic analysis.

Clade	Isolate[a]	Country[b]
1A	LV239, LV3726, LV2750, LV2766, LV4278, LV4361, LV4497	Brazil
1B	LV3213, R83, LV4157, R85	Brazil
1C	LV2948, LV2973	Brazil
1D	LV4102, LV4498	Brazil
1E	L1-130, LV3737	Brazil
1F	LV4211, R62	Brazil
2	2007005490, 2002009669	Hawaii
3	200903008, 200701182	French Polynesia
4	2006006972, 200600682, M20, Wijnberg	Egypt, Denmark, Netherlands
5	Ictero1, Yamamoto, Shibaura	Japan
6	200801909, 201000458, 201100516, 200700457, 200201190	Guadeloupe, Slovenia
7	CIDEIM R006, CIDEIM R107, CIDEIM R081, CIDEIM R103	Colombia

[a,b]*Details of all the isolates and countries of origin are listed in* ***Table S1****.*

Our results indicated that an internal indel in a homopolymeric tract region explains one key aspect of sequence diversity between closely related *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae strains. This observation agrees with previous results where homopolymeric regions were known to be subject to indel mutations as major sources of sequence diversity in various animal, plants, insects and bacteria (Moran et al., 2009). However, indels in homopolymers more often have deleterious effects, and there is an increasing evidence for strong purifying selection against frameshift mutations in coding regions (Williams and Wernegreen, 2013). Therefore, the conserved frameshifting indel in *lic12008* observed in *L. interrogans* serovar Icterohaemorrhagiae isolates is interesting from an evolutionary perspective.

In this study, the power of variant discovery was enhanced by jointly analyzing all the samples (Li and Durbin, 2010; DePristo et al., 2011; Nielsen et al., 2011). Subsequently genotype likelihood-based LRT was assessed to compute a statistically robust association that was able to classify *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae isolates. This test combined with the number of sequences analyzed, demonstrated that both serovars are highly related beyond serovar classification. Thus, the majority of SNPs and indels

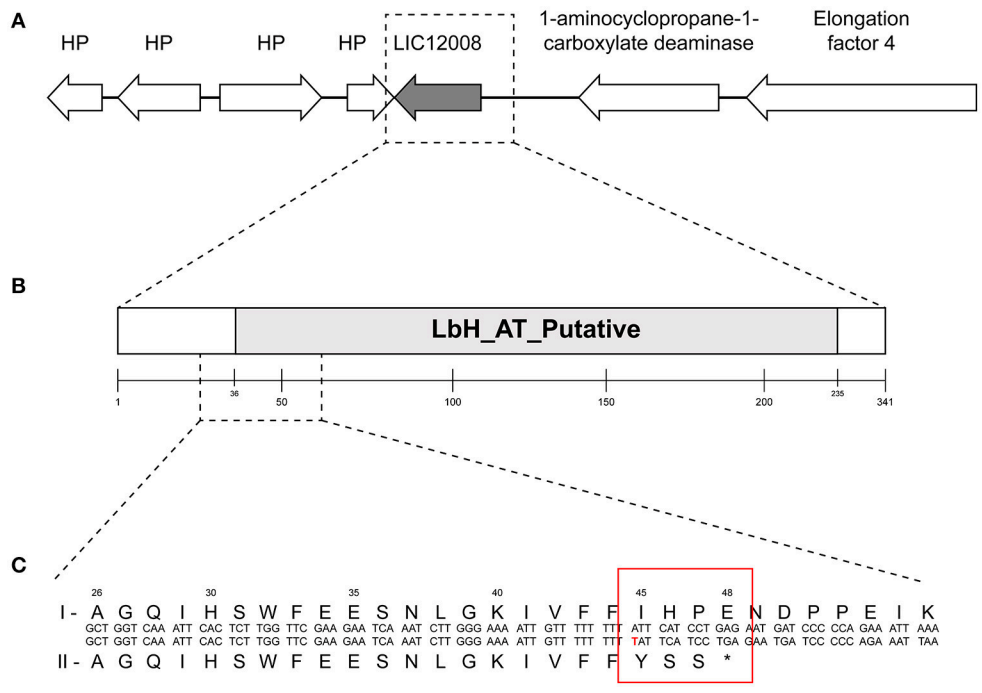


FIGURE 3 | Genomic organization and domain architecture of gene *lic12008*. **(A)** Organization of *lic12008* loci in *L. interrogans* serovars. **(B)** Domain architecture of LIC12008 protein. **(C)** Details of amino acid fragment (1-55aa) showing the sequence differences in *L. interrogans* Copenhageni (I) and *L. interrogans* Icterohaemorrhagiae (II) serovars.

detected among the 67 strains represented the natural diversity of the sequences. In a recent study, MST was employed to differentiate Copenhageni and Icterohaemorrhagiae serovars. However, MST could not define unique profiles for few strains of *L. interrogans* serovars Copenhageni (M20 and Wijnberg strains) and Icterohaemorrhagiae (RGA and Verdun strains) (Zilber et al., 2014). The identified Indel in *lic12008* gene however had a high discriminatory power to distinguish between Copenhageni and Icterohaemorrhagiae serovars without any exceptions.

Analysis of Genomic Region With Predicted Indels

Basic local alignment search tool (BLAST) was used to study the distribution of *lic12008* gene in other *Leptospira* species. Nucleotide analyses demonstrated that this gene was absent in non-pathogenic and intermediate *Leptospira* species and present only in four pathogenic species: *L. interrogans, L. kirschneri, L. noguchii,* and *L. santarosai.* BLAST with the LIC12008 amino acid sequence as query (cutoff: >30% identity) identified UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase, sugar O-acyltransferase and sialic acid O-acetyltransferase of NeuD family as closest homologs. In other bacteria, UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase is involved in the biosynthesis of lipid A, a phosphorylated glycolipid that anchors the lipopolysaccharide to the outer membrane of the cell (Bartling and Raetz, 2009). Previous studies demonstrated the physiological relevance of *lpxD1* gene (LIC13046) encoding a UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase in *L. interrogans* serovar Manilae strain L495 (Eshghi et al., 2015).

We identified a paralog of UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase, annotated as an acetyl transferase gene (LIC12184), in *Leptospira interrogans* Copenhageni Fiocruz L1-130, employing LIC12008 as query sequence (with identity of 74% and similarity of 89%). Further genomic characterization of this paralog might unveil the evolutionary mechanisms underlying the development of this gene family.

Domain analysis of the hypothetical protein encoded by *lic12008* showed that the region spanning amino acids 36 to 233 (out of 242aa) is comprised of the putative Acyltransferase (AT) and Left-handed parallel beta-Helix (LbH) domain (*E*-value: 6.33e-48 and domain accession cd03360) (**Figure 3B**). LIC12008 belongs to LbetaH superfamily proteins composed mainly of acyltransferases [33]. Three imperfect tandem repeats of a hexapeptide repeat motif (X-[STAV]-X-[LIV]-[GAED]-X) were also identified in LIC12008. Thus, the presence of LbHAT domain in LIC12008 (**Figure 3B**) allows us to speculate that this protein has a role in LPS biosynthesis. Previous studies implicated the role of horizontal transfer of genes located within the *rfb* cluster for serological relatedness of genetically similar serovars (de la Peña-Moctezuma et al., 1999; Nalam et al., 2010). In contrast our study identified an indel in *lic12008* gene unrelated to *rfb* cluster but with a presumable role in LPS biosynthesis. Further gene neighborhood analysis of *lic12008* did not show any evidence of horizontal gene transfer events (data not shown).

In clinical strains of *Burkholderia pseudomallei*, accumulation of four indels affecting lipopolysaccharide (LPS) biosynthesis was identified as a mechanism used by this pathogen to evade the immune response (Price et al., 2013). In this context, the presence of a frameshifting indel in *lic12008,* a LPS biosynthesis related gene seems important. Since protective antibody responses for *Leptospira* are against LPS, altered expression of LPS might have an impact on the host immune response, which might provide a plausible explanation for the serological differences found between Copenhageni and Icterohaemorrhagiae strains.

Functional Analysis

Gene expression analysis was performed to identify differences in expression of the *lic12008* transcript in *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae. Two pairs of primers were used to study the impact of mutation on *lic12008* expression. The first primer (12008 T1) encompassed the region of the mutation while the second primer was outside the mutation (12008 T2) (**Figure 3C**, **Table S7**). Transcripts from both regions of *L. interrogans* serovar Icterohaemorrhagiae were downregulated compared to *L. interrogans* Copenhageni Fiocruz L1-130 (**Figure S3**).

To determine the possible phenotypic consequences of the *lic12008* frameshifting indel in *L. interrogans* serovar Icterohaemorrhagiae, we performed an *in vivo* experiment in hamster model of infection. Representative strains from *L. interrogans* serovar Icterohaemorrhagiae were used to infect two groups of three hamsters, via intraperitoneal and conjunctival routes respectively. Both strains of serovar Icterohaemorrhagiae were virulent in the hamster model of infection (**Table S8**) similar to Copenhageni strains which were previously shown to be virulent (Silva et al., 2008). This indicates that *lic12008* frameshifting indel might not be associated with any negative effects on virulence. Alternatively, paralogous gene of *lic12008* might compensate for any functional aberrations caused by this frameshifting indel.

In summary, we analyzed the genome-wide SNPs and indels among the *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae isolates. Analyses of these genome-wide variations revealed that both serovars are genetically similar. Phylogenetic analyses also indicated that *L. interrogans* serovar Copenhageni and Icterohaemorrhagiae strains are highly conserved along time with a distinct geographical clustering. However, our results indicated that an internal indel is the major source of variation in *L. interrogans* serovar Icterohaemorrhagiae. This identified internal indel could provide a plausible explanation for the unexplained antigenic differences between *Leptospira interrogans* serovars Icterohaemorrhagiae and Copenhageni. Previously, indel studies in other species have led to the identification of powerful taxon diagnostics and phylogenetic markers (Baldauf and Palmer, 1993; de Jong et al., 2003; Ajawatanawong and Baldauf, 2013). In this context, the internal indel identified in this study could be possibly validated as a diagnostic marker to differentiate *L. interrogans* Copenhageni and Icterohaemorrhagiae isolates.

AUTHOR CONTRIBUTIONS

LS, AK, EW, and MR designed research. LS and HA performed sample preparation and total DNA extraction. DF and JV performed the sequencing of the isolates. LS and XY analyzed the sequencing data. XY and HZ performed the statistical analyses. LS, LA, and JT performed the evolutionary analyses. HA performed the RT-qPCR experiment and functional analyses. LS and HA drafted the manuscript and also revised the draft. All authors read and approved the final manuscript.

FUNDING

Draft genome sequences used in this study are part of a project which was funded with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract number HHSN272200900007C. This project has also been partially funded by the following grants: R01AI052473 (AK), U01AI088752 (AK), R25TW009338 (AK), R01TW009504 (AK), and R01AI121207 (AK). LS was supported by Programa Ciências sem fronteiras Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) of Brazil and Brasil Sem Miséria Program of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superios (Capes) of Brazil.

ACKNOWLEDGMENTS

We thank the Leptospirosis team at Instituto Gonçalo Moniz, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil, for all the support and technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00193/full#supplementary-material

Figure S1 | Bayesian maximum clade credibility phylogenetic tree. The tree was constructed using the relaxed molecular clock model and Bayesian Skyline Plot population size model. The branch support is given by posterior probability >0.88 and is indicated with an asterisk (*). The tree branches are colored by country.

Figure S2 | MacClade analysis. Identification of the migration relation between strains from different geographic locations. The selected regions were Brazil (BR), United States (US), Slovenia (SL), Egypt (EG) Guyana (GY), Guadalupe (GP), European Union (EU), French Polynesia (FP), Colombia (CO), and Japan (JP).

Figure S3 | Gene expression analysis of *lic12008* in *L. interrogans* serovars. Real-time PCR analysis of *lic12008* transcript in *L. interrogans* serovar Icterohaemorrhagiae strains (201000456 and 201000458) by employing primer pairs (12008 T1 and T2). Fold change was calculated by using the $2^{-\Delta\Delta Ct}$ method. Data was normalized by the amount of *flaB* transcript expressed relative to the corresponding value for *L. interrogans* serovar Copenhageni L1-130 and is expressed as mean ± SD.

Table S1 | General information list of all the *Leptospira interrogans* strains of the serogroup Icterohaemorrhagiae used in this study.

Table S2 | Validation rate for the pipeline call for SNPs.

Table S3 | Validation rate for the pipeline call for Indel.

Table S4 | Sequencing and mapping quality.

Table S5 | List of polymorphic nucleotide sites identified in *L. interrogans* serovars from this study.

Table S6 | List of Insertion and deletions (Indels) identified in *L. interrogans* serovars from this study.

Table S7 | Primers employed for qRT-PCR.

Table S8 | *In vivo* testing of virulence for *L. interrogans* serovar Icterohaemorrhagiae strains.

REFERENCES

Ajawatanawong, P., and Baldauf, S. L. (2013). Evolution of protein indels in plants, animals and fungi. *BMC Evol. Biol.* 13:140. doi: 10.1186/1471-2148-13-140

Baldauf, S. L., and Palmer, J. D. (1993). Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11558–11562. doi: 10.1073/pnas.90.24.11558

Barocchi, M. A., Ko, A. I., Ferrer, S. R., Faria, M. T., Reis, M. G., and Riley, L. W. (2001). Identification of new repetitive element in leptospira interrogans serovar copenhageni and its application to PCR-based differentiation of leptospira serogroups. *J. Clin. Microbiol.* 39, 191–195. doi: 10.1128/JCM.39.1.191-195.2001

Bartling, C. M., and Raetz, C. R. H. (2009). Crystal structure and acyl chain selectivity of *Escherichia coli* LpxD, the N-acyltransferase of lipid a biosynthesis. *Biochemistry* 48, 8672–8683. doi: 10.1021/bi901025v

Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., et al. (2003). Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* 3, 757–771. doi: 10.1016/S1473-3099(03)00830-2

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., et al. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Negl. Trop. Dis.* 9:e0003898. doi: 10.1371/journal.pntd.0003898

de Faria, M. T., de Calderwood, M. S., Athanazio, D. A., McBride, A. J. A., Hartskeerl, R. A., Pereira, M. M., et al. (2008). Carriage of Leptospira interrogans among domestic rats from an urban setting highly endemic for leptospirosis in Brazil. *Acta Trop.* 108, 1–5. doi: 10.1016/j.actatropica.2008.07.005

de la Peña-Moctezuma, A., Bulach, D. M., Kalambaheti, T., and Adler, B. (1999). Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: leptospira interrogans subtype Hardjoprajitno and Leptospira borgpetersenii subtype Hardjobovis. *FEMS Microbiol. Lett.* 177, 319–326. doi: 10.1111/j.1574-6968.1999.tb13749.x

de Jong W. W., Van, M. D., Poux, C., Kappé, G., Van, T. R., and Madsen, O. (2003). Indels in protein-coding sequences of *Euarchontoglires* constrain the rooting of the eutherian tree. *Mol. Phylogenet. Evol.* 28, 328–340. doi: 10.1016/S1055-7903(03)00116-7

DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., et al. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* 43, 491–498. doi: 10.1038/ng.806

Drummond, A. J., and Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7:214. doi: 10.1186/1471-2148-7-214

Eshghi, A., Henderson, J., Trent, M. S., and Picardeau, M. (2015). Leptospira interrogans lpxD homologue is required for thermal acclimatization and virulence. *Infect. Immun.* 83, 4314–4321. doi: 10.1128/IAI.00897-15

Faine, S. (1994). *Leptospira and leptospirosis. Leptospira Leptospirosis.* Available online at: https://www.cabdirect.org/cabdirect/abstract/19952009809 (Accessed Nov 23, 2017). doi: 10.1016/j.vetmic.2009.03.012

Finn, R. D., Tate, J., Mistry, J., Coggill, P. C., Sammut, S. J., Hotz, H.-R., et al. (2008). The Pfam protein families database. *Nucleic Acids Res.* 36, D281–D288. doi: 10.1093/nar/gkh121

Fournier, P.-E., Dubourg, G., and Raoult, D. (2014). Clinical detection and characterization of bacterial pathogens in the genomics era. *Genome Med.* 6:114. doi: 10.1186/s13073-014-0114-2

Galloway, R. L., and Levett, P. N. (2010). Application and validation of PFGE for serovar identification of leptospira clinical isolates. *PLoS Negl. Trop. Dis.* 4:e824. doi: 10.1371/journal.pntd.0000824

Garnier, T., Eiglmeier, K., Camus, J.-C., Medina, N., Mansoor, H., Pryor, M., et al. (2003). The complete genome sequence of *Mycobacterium bovis*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7877–7882. doi: 10.1073/pnas.1130426100

Gutacker, M. M., Smoot, J. C., Migliaccio, C. A. L., Ricklefs, S. M., Hua, S., Cousins, D. V., et al. (2002). Genome-wide analysis of synonymous single nucleotide polymorphisms in *Mycobacterium tuberculosis* complex organisms: resolution of genetic relationships among closely related microbial strains. *Genetics* 162, 1533–1543.

Harrison, F. (2013). Bacterial cooperation in the wild and in the clinic: Are pathogen social behaviours relevant outside the laboratory? *Bioessays* 35, 108–112. doi: 10.1002/bies.201200154

Hillis, D. M., and Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42, 182–192. doi: 10.1093/sysbio/42.2.182

Ido, Y., Hoki, R., Ito, H., and Wani, H. (1917). The rat as a carrier of spirochæta icterohæmorrhagiæ, the causative agent of weil's disease (spirochætosis icterohæmorrhagica). *J. Exp. Med.* 26, 341–353. doi: 10.1084/jem.26.3.341

Johnson, R. C., and Harris, V. G. (1967). Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *J. Bacteriol.* 94, 27–31.

Jordan, I. K., Rogozin, I. B., Wolf, Y. I., and Koonin, E. V. (2002). Microevolutionary genomics of bacteria. *Theor. Popul. Biol.* 61, 435–447. doi: 10.1006/tpbi.2002.1588

Joshi, D., Harris, N. B., Waters, R., Thacker, T., Mathema, B., Krieswirth, B., et al. (2012). Single nucleotide polymorphisms in the *Mycobacterium bovis* genome resolve phylogenetic relationships. *J. Clin. Microbiol.* 50, 3853–3861. doi: 10.1128/JCM.01499-12

Kmety, E., and Dikken, H. (1993). *Classification of the Species Leptospira Interrogans and History of its Serovars*. University Press Groningen.

Ko, A. I., Reis, M. G., Dourado, C. M. R., Johnson, W. D., and Riley, L. W. (1999). Urban epidemic of severe leptospirosis in Brazil. *Lancet* 354, 820–825. doi: 10.1016/S0140-6736(99)80012-9

Ko, A. I., Goarant, C., and Picardeau, M. (2009). Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* 7, 736–747. doi: 10.1038/nrmicro2208

Lehmann, J. S., Matthias, M. A., Vinetz, J. M., and Fouts, D. E. (2014). Leptospiral Pathogenomics. *Pathogens* 3, 280–308. doi: 10.3390/pathogens3020280

Li, H., and Durbin, R. (2010). Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* 26, 589–595. doi: 10.1093/bioinformatics/btp698

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Liu, M., Watson, L. T., and Zhang, L. (2015). Predicting the combined effect of multiple genetic variants. *Hum. Genomics* 9:18. doi: 10.1186/s40246-015-0040-4

Llanes, A., Restrepo, C. M., and Rajeev, S. (2016). Whole genome sequencing allows better understanding of the evolutionary history of Leptospira interrogans serovar Hardjo. *PLoS ONE* 11:e015938710. doi: 10.1371/journal.pone.0159387

Lunter, G., and Goodson, M. (2011). Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. *Genome Res.* 21, 936–939. doi: 10.1101/gr.111120.110

Maddison, W. P., and Maddison, D. R. (1989). Interactive analysis of phylogeny and character evolution using the computer program macclade. *Folia Primatol.* 53, 190–202. doi: 10.1159/000156416

Majed, Z., Bellenger, E., Postic, D., Pourcel, C., Baranton, G., and Picardeau, M. (2005). Identification of variable-number tandem-repeat loci in leptospira interrogans sensu stricto. *J. Clin. Microbiol.* 43, 539–545. doi: 10.1128/JCM.43.2.539-545.2005

Mcbride, A. J. A., Athanazio, D. A., Reis, M. G., and Ko, A. I. (2005). Leptospirosis. *Curr. Opin. Infect. Dis.* 18, 376–386. doi: 10.1097/01.qco.0000178824.05715.2c

Moran, N. A., McLaughlin, H. J., and Sorek, R. (2009). The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. *Science* 323, 379–382. doi: 10.1126/science.1167140

Moreno, L. Z., Miraglia, F., Lilenbaum, W., Neto, J. S., Freitas, J. C., Morais, Z. M., et al. (2016). Profiling of Leptospira interrogans, *L. santarosai*, *L. meyeri* and *L. borgpetersenii* by SE-AFLP, PFGE and susceptibility testing—a continuous attempt at species and serovar differentiation. *Emerg. Microbes Infect.* 5:e17. doi: 10.1038/emi.2016.16

Nalam, K., Ahmed, A., Devi, S. M., Francalacci, P., and Baig, M., et al (2010). Genetic affinities within a large global collection of pathogenic Leptospira: implications for strain identification and molecular epidemiology. *PLoS ONE* 5:e12637. doi: 10.1371/journal.pone.0012637

Nascimento, A. L. T. O., Ko, A. I., Martins, E. A. L., Monteiro-Vitorello, C. B., Ho, P. L., Haake, D. A., et al. (2004). Comparative genomics of two leptospira interrogans serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* 186, 2164–2172. doi: 10.1128/JB.186.7.2164-2172.2004

Nielsen, R., Paul, J. S., Albrechtsen, A., and Song, Y. S. (2011). Genotype and SNP calling from next-generation sequencing data. *Nat. Rev. Genet.* 12, 443–451. doi: 10.1038/nrg2986

Picardeau, M. (2017). Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat. Rev. Microbiol.* 15, 297. doi: 10.1038/nrmicro.2017.5

Price, E. P., Sarovich, D. S., Mayo, M., Tuanyok, A., Drees, K. P., Kaestli, M., et al. (2013). Within-host evolution of *Burkholderia pseudomallei* over a twelve-year chronic carriage infection. *mBio* 4:e00388–13. doi: 10.1128/mBio.00388-13

Puckett, E. E., Park, J., Combs, M., Blum, M. J., Bryant, J. E., Caccone, A., et al. (2016). Global population divergence and admixture of the brown rat (*Rattus norvegicus*). *Proc. R. Soc. B* 283:20161762. doi: 10.1098/rspb.2016.1762

Read, T. D., Salzberg, S. L., Pop, M., Shumway, M., Umayam, L., Jiang, L., et al. (2002). Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. *Science* 296, 2028–2033. doi: 10.1126/science.1071837

Reis, R. B., Ribeiro, G. S., Felzemburgh, R. D. M., Santana, F. S., Mohr, S., Melendez, A. X. T. O., et al. (2008). Impact of environment and social gradient on Leptospira infection in urban slums. *PLoS Negl. Trop. Dis.* 2:e228. doi: 10.1371/journal.pntd.0000228

Ren, S.-X., Fu, G., Jiang, X.-G., Zeng, R., Miao, Y.-G., Xu, H., et al. (2003). Unique physiological and pathogenic features of Leptospira interrogans revealed by whole-genome sequencing. *Nature* 422:888. doi: 10.1038/nature01597

Rogers, J. S., and Swofford, D. L. (1998). A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Syst. Biol.* 47, 77–89. doi: 10.1080/106351598261049

Romero, E. C., Blanco, R. M., and Galloway, R. L. (2011). Analysis of multilocus sequence typing for identification of leptospira isolates in Brazil *J. Clin. Microbiol.* 49, 3940–3942. doi: 10.1128/JCM.01119-11

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Silva, É. F., Santos, C. S., Athanazio, D. A., Seyffert, N., Seixas, F. K., Cerqueira, G. M., et al. (2008). Characterization of virulence of leptospira isolates in a hamster model. *Vaccine* 26, 3892–3896. doi: 10.1016/j.vaccine.2008.04.085

Vinetz, J. M. (1996). Sporadic urban leptospirosis. *Ann. Intern. Med.* 125:794. doi: 10.7326/0003-4819-125-10-199611150-00002

Williams, L. E., and Wernegreen, J. J. (2013). Sequence context of indel mutations and their effect on protein evolution in a bacterial endosymbiont. *Genome Biol. Evol.* 5, 599–605. doi: 10.1093/gbe/evt033

Wunder, E. A., Figueira, C. P., Santos, G. R., Lourdault, K., Matthias, M. A., Vinetz, J. M., et al. (2016). Real-time PCR reveals rapid dissemination of leptospira interrogans after intraperitoneal and conjunctival inoculation of hamsters. *Infect. Immun.* 84, 2105–2115. doi: 10.1128/IAI.00094-16

Zilber, A.-L., Picardeau, M., Ayral, F., Artois, M., Demont, P., Kodjo, A., et al. (2014). High-resolution typing of leptospira interrogans strains by multispacer sequence typing. *J. Clin. Microbiol.* 52, 564–571. doi: 10.1128/JCM.02482-13

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership