January 2022

Leptospiral Proteins As Potential Immunity Targets To Protect Individuals Against Reinfection

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March 17, 2022

Abstract

Leptospirosis is a disease caused by pathogenic species of spirochetes of the genus *Leptospira*. The bacteria are widespread globally and can survive in the environment for weeks after being excreted through the urine of infected animals. Humans get infected through contact with contaminated water or soil. Leptospirosis is a life-threatening disease with a wide range of symptoms. It is believed that after infection, individuals acquire natural immunity against the same infecting serovar. However, there are over 300 serovars of *Leptospira* that can cause disease in humans and animals, and reinfections are common. Nevertheless, recent studies have shown that reinfection caused by the same serovar is frequent in highly endemic areas, indicating that antibodies in different individuals may be diverse. Our research has been focusing on trying to better understand the natural immunity against *Leptospira*. We conducted experiments to verify if antibodies against specific leptospiral proteins could induce immunity against secondary infection. Using mutants and recombinant proteins of the identified targets, we evaluated the role of those protein candidates on the pathogenesis of the bacteria and on the immunity of individuals living in an endemic area for leptospirosis. We identified proteins that have a role as a virulence factor and confirmed the overall role of specific targets as an immunogenic marker for protection. Our preliminary results indicate that those targets can be explored as potential diagnostic and/or prevention candidates against this important neglected disease.

Contents

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. Leptospirosis range from asymptomatic disease to severe cases of liver and renal failure, known as Weil's disease or leptospirosis pulmonary hemorrhage syndrome (LPHS) [1]. It is considered a major public health problem worldwide [2]. The *Leptospira* genus has 68 species with more than 300 serovars and is classified into four phylogenetic subclades: pathogenic P1 and P2 and saprophytic S1 and S2 [3]. *Leptospira* has the ability to colonize the kidney tubules of a wide range of reservoir mammals, with rats being an important one, without causing disease [4]. The bacteria are eliminated by their urine, contaminating the environment. Spill-over infections can occur on susceptible animals, including humans, by contact with the urine or contaminated environment.

Antibodies play an important role in combating the disease[5]. Most antibodies aim at lipopolysaccharides (LPS) of *Leptospira*, which is diverse among various strains [6]. A host can produce agglutinating antibodies after the infection, which are limited to the same serovar of *Leptospira* because of different LPS. For that reason, immune response to one serovar of *Leptospira* could not give individuals cross-protective immunity to other serovars [7]. Nevertheless, recent studies have shown that individuals with mild and severe leptospirosis have a strong immune response against specific leptospiral proteins [8] and that impairment of antibody response can lead to death [9].

We recently performed a case-control study with 126 individuals selected from a longitudinal cohort of >2,000 individuals living in a slum area in Salvador, Brazil, an endemic region for leptospirosis. We studied the hypothesis that pre-exposure inversely correlates with the risk of infection, and antibodies anti-proteins recognized in sera are markers for protection. Based on serology, we selected individuals with one or more reinfection events (cases) and individuals with

only one infection (controls). Individuals were matched by age, gender, time of collection of sera, same follow-up, and same recruitment time. Using the proteome array approach in collaboration with UC Irvine [7, 8], we identified 37 unique proteins (IgM and IgG responses) that are related to potential protection against reinfection. Of those, 18 (48.6%) were also identified in our attenuated vaccine model[7], indicating the potential role of protection. For this present study, we characterized the role of some of those protein targets related to the pathogenesis and immunity of the pathogen. We evaluated the virulence phenotype of mutants lacking the expression of specific protein targets and characterized the immune response of individuals with and without reinfection events to those proteins using an ELISA approach. Studies to better characterize these protein candidates and understand their role in the immunity against leptospirosis or the biology of the agent will help to close the gap regarding naturally acquired immunity and the development of improved prevention and diagnostic methods.

Specific Aim & Hypotheses

This project aims to validate protein candidates that correlate with immune protection against reinfection in humans and have the potential to improve diagnostic and prevention.

1) We hypothesize that the deletion of one of the protein candidates will affect the virulence of the strain, indicating a role in the pathogenesis of the disease and highlighting the potential of the specific protein to be used as a surrogate for protection.

2) We hypothesize that using those proteins in an ELISA platform, we can identify individuals that had only one infection event, indicating the potential of those proteins to be used as surrogates for immunity and potential diagnostic markers.

Material and Methods

Leptospira spp. strains and culture

Mutant strains were selected from a library of random mutants generated with *Himar1* transposon. Frozen aliquots of mutants of interest have been kept at -80 ℃. The insertion of the transposon and confirmation of gene disruption was done using a semi-random PCR. Of the 37 genes that were identified in our case-control study, 11 (30%) had at least one mutant that could be evaluated (Table 1). All mutants were generated from *L. interrogans* serovar Manilae parent strain (wildtype). The frozen aliquots of mutants or wild type of interest were thawed in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) [10] and kept at 29°C for 14 days. After checking for growth, motility, and lack of contamination, the strains were kept in liquid EMJH, culturing every week for up to 5-6 weeks, until animal studies. For the challenge experiment, the concentration of leptospires was determined under a dark-field microscope using a Petroff-Hausser counting chamber (Fisher Scientific).

Table 1. Genes of interest with mutant strains and/or recombinant protein to evaluate pathogenesis and immunity

Gene ID	Localization	Mutant	Ig	Recombinant protein
LIC10050	Outer membrane	Yes	IgM	Yes
LIC10465	Outer membrane	Yes	IgM	Yes
LIC11845	Cytoplasmic	Yes	IgG	Yes
LIC11941	Outer membrane	Yes	IgG and &IgM	Yes
LIC10010	Unknown	Yes	IgM	No
LIC10080	Cytoplasmic	Yes	IgM	No

LIC10491	Cytoplasmic	Yes	IgM	No
LIC10544	Unknown	Yes	IgG	No
LIC10829	Extracellular	Yes	IgG	No
LIC11510	Cytoplasmic	Yes	IgM	No
LIC20152	Unknown	Yes	IgG	No
LIC11019	Unknown	No	IgG and IgM	Yes
LIC11073	Extracellular	No	IgG	Yes
LIC11186	Outer membrane	No	IgG	Yes
LIC11623	Outer membrane	No	IgG	Yes
LIC11694	Outer membrane	No	IgG	Yes
LIC12544	Unknown	No	IgG	Yes
LIC13084	Outer membrane	No	IgG	Yes
LIC20016	Cytoplasmic	No	IgG and IgM	Yes

Virulence experiments

The identified mutants were characterized in the hamster model for leptospirosis. Golden Syrian Hamsters are highly susceptible to leptospirosis and are the model of choice for acute leptospirosis, emulating the natural history and clinical presentation of severe leptospirosis in humans [11-13]. Three-week-old hamsters were infected with high doses of the spirochete (10^8 leptospires) through the intraperitoneal (IP) or conjunctival (CJ) route. A group challenged by the same routes with the wild-type strain was kept as a control. Animals were observed daily for clinical signs up to 21 days post-challenge. Surviving animals at the end of the experiment or moribund animals presenting with difficulty moving, breathing, or signs of bleeding or seizure were immediately sacrificed by inhalation of CO_2. All animal protocols were approved by the Institutional Committee for the Use of Experimental Animals, Yale University (protocol # 2020-11424). All virulence experiments

were conducted twice for reproducibility. Kidneys were obtained during the euthanasia for further analysis.

DNA extraction and qPCR

To evaluate the ability of the mutants to cause renal colonization, we extracted kidney DNA to evaluate by qPCR. DNA was extracted from the kidney cortex using Maxwell® 16 tissue DNA purification kit (Promega Corporation, Madison, WI), following the manufacturer's instructions and using a 200 μL elution volume. The concentration of leptospires was quantified by a TaqMan-based quantitative-PCR assay using an ABI 7500 system (Thermo Fisher Scientific, Inc.) and Platinum Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Inc.). The qPCR reaction was performed using *lipL32* primers and probes as previously described, with the bacterial quantification being calculated and expressed as the number of leptospires per milliliter based on a standard curve [14].

ELISA

According to our case-control study, each target identified was related to IgG and/or IgM response (Table 1). From our 37 protein candidates, we had recombinant protein from 12 (32.5%) of them (Table 1). To validate the immunogenicity of those protein targets in human sera, we tested recombinant proteins using an ELISA assay. To determine the best conditions for the assay, we evaluated the temperature for coating (room temperature and 37°C) and different concentrations of the protein-based on previous experiments: 50 ng, 100 ng, and 200 ng. We also evaluated the best dilution of the HRP anti-human secondary antibody: 1:10,000 and 1:25,000. The primary antibody was used at a dilution of 1:50. For the optimization assay, we used one protein for IgM

(LIC10050) and one for IgG (LIC12544). Protein concentration and quality were measured by Bradford assay and SDS-PAGE, respectively.

We then selected ten random sera samples from each group of our case-control study to create a sera pool. The negative control (NTC) sera used was a commercial normal human serum. As a positive control, we used acute and convalescent sera from leptospirosis patients for IgM and IgG, respectively. We used the sera pool to validated the results of the proteome array using an ELISA assay. Proteins were individually coated in 96-well plates at pre-determined concentrations and evaluated. After using the sera pool to test all proteins (Table 1), we evaluated the recombinant LIC 20016 protein (IgM and IgG) using individual sera samples from our individuals enrolled in our case-control study.

Results

Virulence evaluation

In all virulence evaluation experiments, hamsters challenged with the wildtype strain, either by IP or CJ routes, all died as expected. All mutant strains were evaluated twice using the hamster model. Mutants ΔLIC10465, ΔLIC10491, ΔLIC11941, and ΔLIC10080 were considered virulent since mortalities were 100% for both IP and CJ challenged experiments. The remaining mutants (63.7%) were considered attenuated at different levels, specifically when using the CJ route. Only one mutant, ΔLIC10050, an outer membrane protein (OMP), was attenuated by IP and CJ route. Nevertheless, all of the attenuated mutants were unable to cause death on all hamsters when challenged by CJ route (Table 2).

Table 2. Mortality for IP and CJ route with 95% confidence interval

Strains	Localization	IP mortality (95%CI)	CJ mortality (95%CI)	Attenuated
LIC10050	Outer membrane	33.33% (0.10, 0.7)	16.67% (0.03, 0.56)	Yes
LIC10544	Unknown	100% (0.61, 1)	16.67% (0.03, 0.56)	Yes
LIC20152	Unknown	100% (0.61, 1)	16.67% (0.03, 0.56)	Yes
LIC11510	Cytoplasmic	100% (0.61, 1)	16.67% (0.03, 0.56)	Yes
LIC10829	Extracellular	100% (0.61, 1)	33% (0.10,0.7)	Yes
LIC11845	Cytoplasmic	100% (0.71, 1)	66.67% (0.35, 0.88)	Yes
LIC10010	Unknown	100% (0.80, 1)	73% (0.48, 0.89)	Yes
LIC10465*	Outer membrane	100% (0.61, 1)	100% (0.61, 1)	No
LIC10491	Cytoplasmic	100% (0.61, 1)	100% (0.61, 1)	No
LIC11941	Outer membrane	100% (0.61, 1)	100% (0.61, 1)	No
LIC10080	Cytoplasmic	100% (0.61, 1)	100% (0.61, 1)	No

* Mutant LIC10465 was evaluated by a different member of our group.

Kidneys were collected from hamsters after euthanasia. DNA was then extracted from the kidney and we performed qPCR to determine renal colonization. Results of qPCR showed that despite the attenuation phenotype, the mutants were still able to cause colonization on hamsters (Figure 1). However, compared to wildtype, only three mutant strains had no statistically difference in the renal burden. Despite the lack of attenuation when using the IP route, mutants ΔLIC11510, ΔLIC10491, and ΔLIC10829 had significant difference among IP route (Appendix S1). Furthermore, five of the seven mutants with attenuation on the conjunctival route also showed reduced renal burden. Interestingly, the only mutant that was attenuated on both challenge routes, LIC10050, had no differences on the renal burden. Nevertheless, these results confirm the role of some of those targets on the pathogenesis of the leptospiral agent.

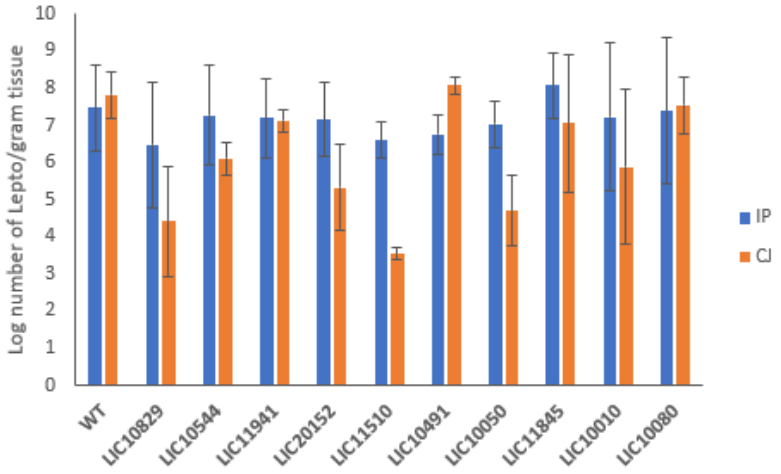

Figure 1. qPCR results for IP route and CJ route. Each bar represents the mean result for the bacterial load (logarithmic scale) of two independent experiments. The error bars represent the standard deviations.

ELISA evaluation

In order to test the optimal condition of ELISA, we performed an optimization assay to determine the best concentration of the antigen, best temperature for coating, and best dilution for the secondary antibody. Our results showed that for both IgM and IgG, the optimal condition was coating with 50 ng of protein and 1:10,000 secondary antibodies at 37 ℃ (Figure 2).

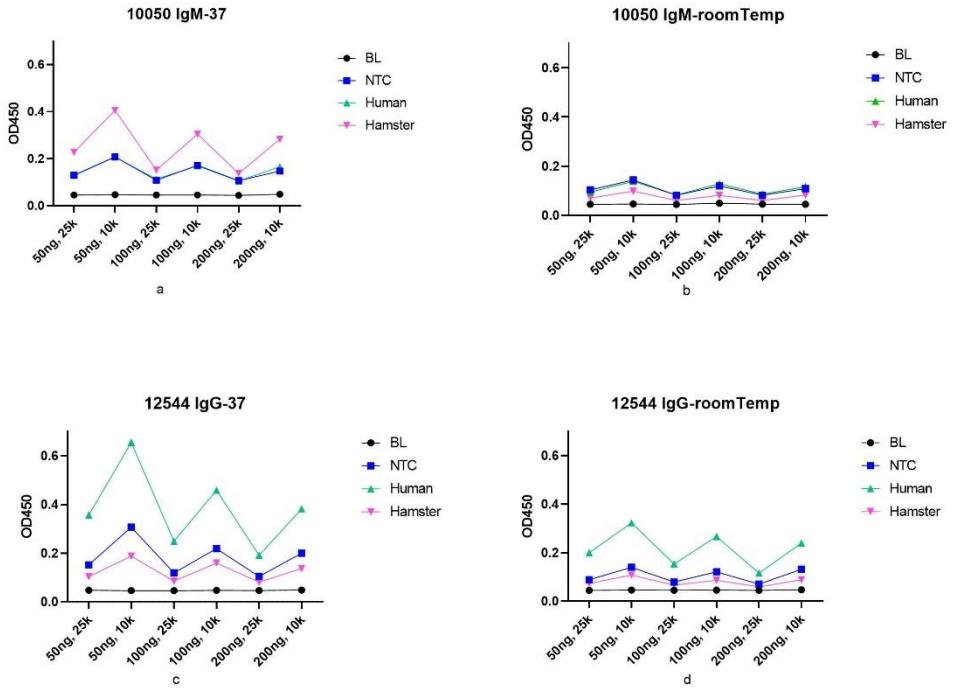

Figure 2. Optimization of ELISA assay. We evaluated the coating using 50ng, 100ng, 200ng protein and the 1:10,000 and 1:25,000 for dilution of the secondary antibody. BL was the blank control using 3% BSA solution; NTC was negative control using commercial NHS sera; Human group used sera from acute leptospirosis patients for IgM and sera from leptospirosis patients in convalescence period for IgG. Hamster group used sera collected from Leptospira infected hamsters. The rLIC10050 was evaluated using anti-IgM secondary antibody with coating at 37 ℃ (a) and Room Temperature (b). The rLIC12544 was evaluated using anti-IgG secondary antibody with coating at 37 ℃ (c) and Room Temperature (d).

ELISA was conducted with all proteins available (Table 1), testing for IgG and/or IgM using a pool of cases and a pool of controls, together with positive and negative controls as described. Results showed that there was a significant difference between the antibodies in the sera pool of the case group and control group for all proteins using IgM, while the difference between the case group and control group for all proteins using IgG was not significant (Figure 3).

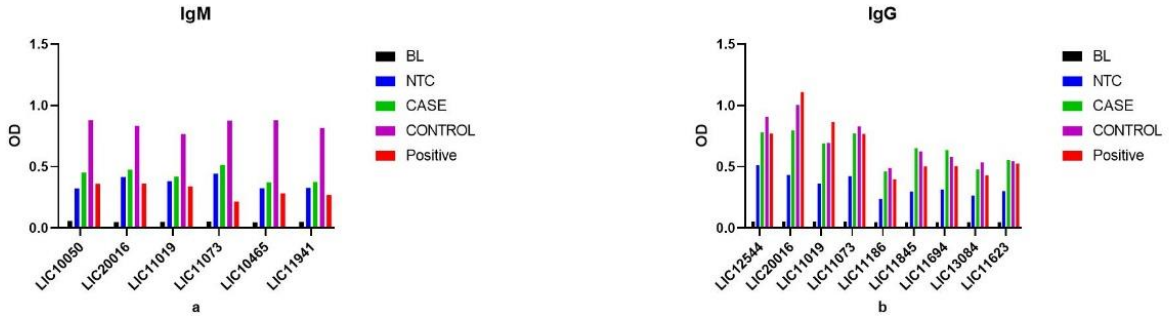

Figure 3. ELISA test using sera pool for IgM and IgG. BL was the blank control using 3% BSA solution; NTC was negative control using commercial NHS sera; CASE was the case group in the case-control study; CONTROL was the control group in the case-control study; Positive used sera from acute leptospirosis patients for IgM and sera from leptospirosis patients in convalescence period for IgG. a) ELISA of LIC10050, LIC20016, LIC11019, LIC11073, LIC10465, and LIC11941 using IgM under the optimal ELISA condition; b) ELISA of LIC12544, LIC20016, LIC11019, LIC11073, LIC11186, LIC11845, LIC11694, LIC13084, and LIC11623 using IgG under the optimal ELISA condition

We then evaluated the recombinant LIC20016 protein with all individual samples. In our case-control study, we evaluated 57 cases and 57 controls. Individual ELISA showed little difference between the case group and control group for both IgM and IgG secondary antibodies. The difference between the means of the case group and the control group was not statistically significant. The standard deviation of the case group was 0.251 for IgM secondary antibody, and it was 0.154 for the control group. For IgG, the SD_{case} was 0.196, and $SD_{control}$ was 0.141. For both IgM and IgG experiments, the standard deviation of the case group was greater than that of the control group.

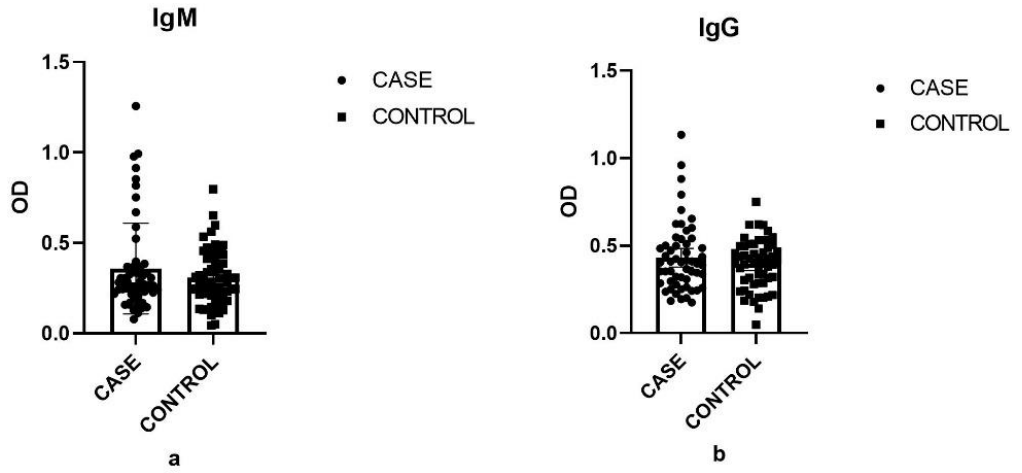

Figure 4. Individual ELISA with rLIC20016: a) ELISA using IgM as the secondary antibody; b) ELISA using IgG as the secondary antibody

Discussion

Humans acquire immunity against leptospirosis to a certain degree after the infection, and the importance of antibodies in the development of this immunity has been demonstrated [15, 16]. There has been an increased effort to develop vaccines that can elicit cross-immunity to prevent leptospirosis on different epidemiological settings. Here, we designed a series of experiments to study the potential value of certain leptospiral proteins on the development of vaccines and diagnostic tools.

The virulence evaluation showed that out of 11 mutants evaluated, seven of them were attenuated at some level when compared to the wildtype strain. More specifically, the attenuation seemed to be more evident when using the conjunctival route of infection. This route is important because it mimics the natural process of infection, where the strain needs to adhere, penetrate and disseminate before causing infection. Those steps are somehow lost during the IP challenge, which highlights the importance of this route and the potential role of those targets during the initial steps of

infection. Despite the attenuation phenotype, all mutants were able to cause renal colonization. However, the burden of leptospires on the kidney cortex was significantly different, confirming the survival results. In other words, some of these proteins play an important role in the infection of *Leptospira*. Leptospira proteins can be unique and the proteome sometimes redundant, with the function of many of them still unclear. Even though they are not completely attenuated, they still contribute to the study of pathogenesis and target research. The deletion of the LigA protein (LIC10465) alone can't affect the virulence, but when the expression of both LigA and LigB are disrupted, the strain becomes attenuated[17, 18]. Given our results, these proteins can be used as potential targets for the design of vaccines and the study of pathogenesis.

It is important to discuss that, in our experiments, we used a high dose of *Leptospira* to challenge the hamsters. The hypothesis was that the lack of expression of one of those targets would lead to a complete attenuation of the bacterium. However, given our results showing different levels of attenuation, it would be appropriate to perform a LD_{50} experiment with each mutant strain on both routes of challenge, to determine if the attenuation could be related to lower doses of infection. The next stage of experiments should also include the complemented strains of the mutants to confirm the effect of virulence.

Our ELISA results revealed that the sera pool from individuals who got infected only once had more reaction with proteins related to IgM immune response than the sera pool from individuals who got more than once infection. However, there was no significant difference between the sera pool from the case group and the control group when using proteins related to IgG response. When thinking about diagnostic, especially early diagnostic, our results are optimistic since IgM is the first antibody response after infection. Those targets can potentially be used to improve the diagnostic of leptospirosis specially in endemic regions where reinfections are common. The

results with the IgG targets need to be better explored in the future. We only selected ten sera samples randomly to make the sera pool. The pool strategy was used given the large number of protein targets and individuals to be tested. It is possible that by testing a pool with all individuals the results would be different and more accurate, given that the immune response to those targets vary among individuals. It is also possible that the IgG response to individual proteins might need better optimization. We only used one specific protein as representative of each Ig and potentially each protein may have its own specific optimal condition.

While performing ELISA using one specific protein (rLIC20016) to evaluate the sera from our case-control study individually, the proteome array results could not be validated. Despite this protein target being selected as a marker for both IgM and IgG responses, the difference between the case group and control group was not statistically significant for neither when using the ELISA platform. The proteome array method is more sensitive than an ELISA assay, and for that reason some of the targets selected on our high-throughput system might not be validated using a less sensitive method. Unfortunately, we only had time to validate one protein and further analysis of each individual proteins needs to be performed to better understand the role of each target on the immunoprotection against reinfection.

Our preliminary results indicate that some of those targets can be potentially used to improve prevention and the diagnostic of leptospirosis. Evaluating their function and their role on the pathogenesis and the immunity of the disease can contribute to our better understanding of the natural immunity against leptospirosis.

Acknowledgments

I really appreciate the help from Dr. Elsio Wunder Jr. His patience and encouragement inspire me to keep going forward. Thanks to Professor Christian Tschudi for his kind encouragement and guidance in my career. Thanks to Dr. Karukriti Ghosh as my great mentor. She gave me lots of support to help me finish my project. I also want to thank Cate Muenker as our lab technician. She taught me a lot of lab skills in the lab. I could not finish my project without her help. She always believed in me and empowered me, which gave me great confidence. Thanks to Felipe Passalia and Leandro Garcia. They gave me so much great ideas when I faced problems. Lastly, thanks to Dr. Albert Ko for offering me such an opportunity to work here.

Reference:

1. Costa, F., et al., *Global Morbidity and Mortality of Leptospirosis: A Systematic Review.* PLoS Negl Trop Dis, 2015. **9**(9): p. e0003898.
2. McBride, A.J., et al., *Leptospirosis.* Curr Opin Infect Dis, 2005. **18**(5): p. 376-86.
3. Vincent, A.T., et al., *Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics.* PLoS Negl Trop Dis, 2019. **13**(5): p. e0007270.
4. Levett, P.N., *Leptospirosis.* Clin Microbiol Rev, 2001. **14**(2): p. 296-326.
5. Fraga, T.R., A.S. Barbosa, and L. Isaac, *Leptospirosis: aspects of innate immunity, immunopathogenesis and immune evasion from the complement system.* Scand J Immunol, 2011. **73**(5): p. 408-19.
6. Limothai, U., et al., *The role of leptospiremia and specific immune response in severe leptospirosis.* Sci Rep, 2021. **11**(1): p. 14630.
7. Wunder, E.A., et al., *A live attenuated-vaccine model confers cross-protective immunity against different species of the Leptospira genus.* Elife, 2021. **10**.
8. Lessa-Aquino, C., et al., *Distinct antibody responses of patients with mild and severe leptospirosis determined by whole proteome microarray analysis.* PLoS Negl Trop Dis, 2017. **11**(1): p. e0005349.
9. Lindow, J.C., et al., *Cathelicidin Insufficiency in Patients with Fatal Leptospirosis.* PLoS Pathog, 2016. **12**(11): p. e1005943.
10. Johnson, R.C. and V.G. Harris, *Differentiation of pathogenic and saprophytic letospires. I. Growth at low temperatures.* J Bacteriol, 1967. **94**(1): p. 27-31.
11. Randall, R. and H.K. Cooper, *The Golden Hamster (Cricetus Auratus) as a Test Animal for the Diagnosis of Leptospirosis.* Science, 1944. **100**(2589): p. 133-4.

12. van den Ingh, T.S. and E.G. Hartman, *Pathology of acute Leptospira interrogans serotype icterohaemorrhagiae infection in the Syrian hamster.* Vet Microbiol, 1986. **12**(4): p. 367-76.
13. Strauss, A.L., et al., *Doppler ophthalmic blood pressure measurement in the hemodynamic evaluation of occlusive carotid artery disease.* Stroke, 1989. **20**(8): p. 1012-5.
14. Wunder, E.A., Jr., et al., *Real-Time PCR Reveals Rapid Dissemination of Leptospira interrogans after Intraperitoneal and Conjunctival Inoculation of Hamsters.* Infect Immun, 2016. **84**(7): p. 2105-2115.
15. Adler, B., et al., *Maturation of humoral immune response determines the susceptibility of guinea-pigs to leptospirosis.* Pathology, 1980. **12**(4): p. 529-38.
16. Inada, R., et al., *The Etiology, Mode of Infection, and Specific Therapy of Weil's Disease (Spirochaetosis Icterohaemorrhagica).* J Exp Med, 1916. **23**(3): p. 377-402.
17. Ko, A.I., C. Goarant, and M. Picardeau, *Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen.* Nat Rev Microbiol, 2009. **7**(10): p. 736-47.
18. Picardeau, M., *Virulence of the zoonotic agent of leptospirosis: still terra incognita?* Nat Rev Microbiol, 2017. **15**(5): p. 297-307.

Appendix

S1. T-test for the difference of qPCR between wild type and mutant strains by IP and CJ route

Mutant strains	P-value for IP route	P-value for CJ route
LIC10829	<0.01*	<0.01*
LIC10544	0.691	<0.01*
LIC11941	0.574	<0.01*
LIC20152	0.514	<0.01*
LIC11510	<0.01*	0.025*
LIC10491	0.022*	0.141
LIC10050	0.071	0.129
LIC11845	0.151	0.533
LIC10010	0.273	0.008*
LIC10080	0.585	0.370

S2. Information on Gene ID, name, and localization with Ig

Gene ID	Product name	Localization	Ig
LIC20152	Hypothetical protein	Unknown	IgG
LIC10829	Hypothetical protein	Extracellular	IgG
LIC11623	Outer membrane protein	Outer Membrane	IgG
LIC11019	Putative Lipoprotein	Unknown	IgG& IgM
LIC13084	Hypothetical protein	Unknown	IgG
LIC11186	Putative Flagellar protein	Outer Membrane	IgG
LIC11845	Hypothetical protein	Unknown	IgG
LIC10544	Outer membrane protein	Unknown	IgG

LIC11073	Putative Lipoprotein	Unknown	IgG& IgM
LIC12544	DNA binding protein	Unknown	IgG
LIC20016	Hypothetical protein	Cytoplasmic Membrane	IgG& IgM
LIC10491	Acriflavin resistance	Cytoplasmic Membrane	IgM
LIC10010	Hypothetical protein	Unknown	IgM
LIC10465	Partial Ig-like repeat-containing protein	Unknown	IgM
LIC11510	Heavy metal efflux pump	Cytoplasmic Membrane	IgM
LIC10050	Peptidoglycan-associated cytoplasmic membrane protein	Outer Membrane	IgM
LIC11941	Heavy metal efflux pump	Outer Membrane	IgM
LIC10080	Hypothetical protein	Unknown	IgM