EQUINE LEPTOSPIROSIS: A STUDY OF *LEPTOSPIRA INTERROGANS* SEROVAR BRATISLAVA PATHOGENICITY IN HORSES AND DEVELOPMENT OF A DIAGNOSTIC TEST USING REVERSE VACCINOLOGY

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ABSTRACT

Leptospirosis is a neglected worldwide zoonotic disease caused by pathogenic gram-negative spirochetes of the genus *Leptospira*. It can be life threatening for animals and humans. *Leptospira* can cause livestock losses among pigs, cattle, horses, goats and sheep, thereby inflicting significant losses on the livestock agronomy. This is the first time an analysis using *L. interrogans* serovar Bratislava, which is believed to be pathogenic for horses, is presented. Six young female foals were challenged with 1 x 10^9 *L. interrogans* serovar Bratislava strain PigK151 and observed over a period of 30 days. Temperature, blood and urine, as well as post-mortem samples were evaluated. No pyrexia was noted. PCR was negative from all plasma, urine and tissue samples. No Leptospires were recovered from either plasma or urine culture. All challenged foals developed antibodies against *L. interrogans* serovar Bratislava as determined by the gold standard microscopic agglutination test (MAT), beginning on day 3 until the last day of the study while all the control animals remained serologically negative. Cross-reactivity on the MAT test results and DNA analysis helped to formulate a reasonable answer for the question about *L. interrogans* serovar Bratislava pathogenicity in horses. Cross-reactivity among serovars used on the MAT test shows that a better diagnostic test is necessary. Reverse vaccinology has been broadly used to screen for surface-exposed proteins and antigens of important pathogens, like outer membrane proteins and extracellular proteins used as potential vaccine candidates. In

order to develop a test that can serve as a diagnostic test and possibly identify the infecting serovar, similar to the gold standard MAT, a reverse vaccinology approach to identify unique proteins among different serovars proved to be a promising strategy. A previous work identified 861 unique proteins among 17 different strains of pathogenic *L. interrogans*. A subset of these proteins were selected for further study according to their antigenic score, potential B epitopes and size. Eight new leptospiral proteins were cloned, expressed, purified and screened in a serological test along with another four “shared” proteins from *L. interrogans*. Using these new leptospiral antigens, a new enzyme-linked immunosorbent assay (ELISA) was developed and evaluated for diagnosis of equine leptospirosis. DNA analysis showed that some of these unique proteins (5/8) were only present on reported pathogenic strains and species, suggesting that these proteins might play a role in pathogenicity. Four of the eight new leptospiral antigens presented the very promising results. LA_0711 showed a sensitivity (Se) of 100% and a specificity (Sp) of 69%, LA_1567 results were Se = 98% and Sp = 72%, while LIC11823 values were Se = 98% and Sp = 63% and LEP1GSC077_3193 with Se=97% and Sp = 75%. All antigens but one (WP_061243705), differentiate MAT positive from negative samples (P-value<0.05). Multi-antigen combinations were also tested with the four-most accurate antigens (LA_0711, LA_1567, LIC11823 and LEP1GSC077_3193). ELISA results for these four leptospiral antigens were combined in series and in parallel. In a series approach, a tested sample is positive when it is reactive to all proteins it is tested for. Here, a sensitivity of 95% and specificity of 94%

were obtained for series testing for the combination of LA_0711 + LIC11823 + LEP1GSC077_3193. When a parallel method was applied, meaning that a sample considered positive must be reactive to at least one antigen in the combination (combination of two, three or four antigen results), results from LA_1567 and LEP1GSC077_3193 showed the best sensitivity of 99% and specificity of 63%. These single and multi-antigen combinations should be able to supplement if not replace the current MAT for the diagnosis of equine leptospirosis in the near future, after further validation with more equine serum samples that have a more detailed information as clinical phase or identification of infecting serovar/strain done by culture or genetic confirmation.

BIOGRAPHICAL SKETCH

Tiago Jaquel Zilch was born in São Miguel do Iguaçu, Paraná state in Brazil, on June 18, 1991. In 2013 Tiago started his Bachelor in Chemical Engineering at the College of Science and Technology of Viçosa - Univiçosa. He collaborated with Drs. Marcia Lamego's group at the Federal University of Viçosa -UFV during 2013-15, working on the development of a vaccine for *Porcine Circovirus* 2 (PVC2), a swine pathogen. This collaboration resulted in many patents that are deposited internationally in many countries. Tiago's primary role was to express and purify the recombinant protein and produce the vaccine, and to study its stability over a year period. Later on, this vaccine was sold to a Brazilian company, OuroFino Saude Animal, Tiago was subsequently involved in the technology transfer between academia and the company.

In 2016 Tiago was invited to come to Dr. Yung-Fu Chang's laboratory, at Cornell University, to work on the development of another vaccine, against *Mycoplasma Hyopneumoniae,* where many vaccine candidates were developed and are currently under testing in Brazil. In 2017, Tiago went back to Brazil to finish his BS in Chemical Engineering, returning to the United States in August 2018 to pursue his Master of Science degree with Dr. Yung-Fu Chang, this time working on leptospirosis and helping the field with new discoveries that are presented in this thesis. Tiago successfully earned his MS degree in Animal Science in 2020, with minors in Infectious Diseases and Human & Animal Epidemiology.

I dedicate this thesis to the most important people of my life: my mother Glaci, my sisters Talita and Talia, and to my beloved Carl. I am thankful to all of them for supporting me all the way through, for the advice and encouraging words. I also dedicate this thesis to all of the minority people and underrepresented groups in science; let us show the world that we are here and we are standing among the best.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The pathogenic gram-negative spirochete, *Leptospira* spp., is the causative agent of leptospirosis, a bacterial disease generally transmitted to humans and animals through environmentally; usually via contact with water or soil contaminated by the urine of carrier animals or free-living bacteria [1, 2]. In many tropical regions that suffer from flooding, leptospirosis is endemic and a potentially fatal zoonosis [3]. In humans, it is estimated that leptospirosis is responsible for more than 1 million cases and close to 60,000 deaths worldwide annually, with prevalence being higher among males at age 20-49 years [4]. South and Southeast Asia, Oceania, Caribbean, Andean, Central and Tropical Latin America, and East Sub-Saharan Africa present the highest morbidity and mortality rates for leptospirosis [3]. In humans, the symptoms range from a mild flu-like sickness to severe Weil's disease characterized by multi-organ failure, and in some cases leading to death [5, 6]. In animals, especially in horses, some serovars are associated with many clinical syndromes, including: pyrexia [7] uveitis [8, 9], abortion [10, 11], recurrent uveitis [12, 13] and renal failure [14]. The improvement of leptospirosis diagnostic testing as well as the development of new vaccines able to stimulate protective host response, preventing subclinical infections in carriers, are urgently needed. This chapter presents a literature review on *Leptospira*, pathogenicity, epidemiology and leptospirosis diagnostic tests.

Bacteriology of Leptospires

Leptospires are best visualized using dark field microscopy, with cell diameter of usually 0.1 μm, length range of 6 to 20 μm, but some *in vitro* cultures may contain much longer cells. These are tightly coiled spirochetes, with helical amplitude is approximately 0.1 to 0.15 μm. One noticeable characteristic of these spirochetes is that the cells have pointed ends, on either or both sides, of which are usually bent into a distinctive hook, resembling a question mark as shown in Figure 1 [15]. Morphologically, all leptospires are indistinguishable *in vivo*, but *in vitro* subculture morphology varies, but it can be restored by passage in hamsters as well as restoring virulence [16].

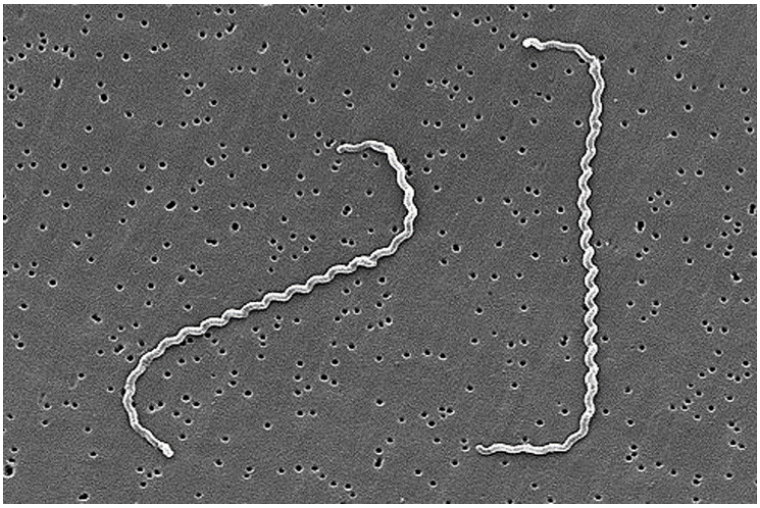

Figure 1.1: Scanning electron microscopic (SEM) image of *L. interrogans* strain-RGA bacteria. Obtained from the CDC Public Health Image Library. Image credit: CDC/NCID/HIP/Janice Carr (PHIL #1220)

Leptospira, as other spirochetes, have a distinctive double-membrane architecture, but *Leptospira* shares characteristics of both gram-positive and gram-negative bacteria. This genus features a double membrane structure in which the cytoplasmic membrane is closely associated with the peptidoglycan cell wall resembling gram-positive bacteria. In addition, leptospires also have an outer membrane that provides a barrier protecting underlying antigens, such as the endoflagella, from elements of host immune system [17]. Nevertheless, the leptospiral outer membrane appears to be fluid and labile, contrasting it with the outer membrane of gram-negative bacteria.

Unlike most other pathogenic spirochetes, the leptospiral outer membrane is rich in lipopolysaccharide (LPS), similar to that of gram-negative bacteria, but this bacterium has a number of unique structural features. LPS is a major component of the leptospiral outer membrane and is the principal antigen that the humoral immune response targets during infection [18]. Leptospiral LPS consists of three components: lipid A, the core, and polysaccharide, which has a much lower endotoxic activity compared to other gram-negative bacteria due to changes on its lipidA structure [18-21]. These changes may explain why leptospiral LPS is not recognized by the LPS-specific Toll-like receptor 4 of humans. Indeed, the differences among leptospiral LPS structures are the basis for the categorization of *Leptospira*.

Classification

The genus *Leptospira* has recently been redefined, being now composed of 66 different species that include more than 300 serovars [22-25]. Phylogenetic analysis revealed that *Leptospira* can be divided in three lineages that correlate with the level of pathogenicity of the species: saprophytic, intermediate, and pathogenic [26, 27]. The updated data from *Leptospira* classification is shown in Table 1.1.

Table 1.1: Classification and data available from the 66 *Leptospira* species.

Species	Virulence	Strain (N)	Genomes (N)	Geographic origin	Source of isolation	Reference
L. adleri	pathogenic	3	3	Africa - Oceania	environmental	(Thibeaux *et al*., 2018a)
L. alexanderi	pathogenic	13	6	Asia	bovine - hedgehog - human	(Brenner *et al*., 1999)
L. alstonii	pathogenic	5	5	Asia	amphibians	(Brenner *et al*., 1999; Smythe *et al*., 2013)
L. andrefontaineae	probable intermediate	2	1	Oceania	environmental	(Vincent *et al*., 2019)
L. bandrabouensis	probable saprophytic	3	2	Africa	environmental	(Vincent *et al*., 2019)
L. barantonii	pathogenic	2	2	Asia - Oceania	environmental	(Thibeaux *et al*., 2018a)
L. biflexa	saprophytic	9	7	Asia - Europe - North America	environmental - human	(Faine and Stallman, 1982; Yasuda *et al*., 1987)
L. borgpetersenii	pathogenic	220	60	Africa - Asia - Europe - North America - Oceania - South America	amphibians - bandicoot - bovine - canine - equine - hedgehog - human - opossum - porcine - roden - shrew	(Yasuda *et al*., 1987)
L. bourretii	probable saprophytic	5	4	Oceania	environmental	(Vincent *et al*., 2019)
L. bouyouniensis	probable saprophytic	4	3	Africa - Oceania	environmental	(Vincent *et al*., 2019)
L. brenneri	probable saprophytic	2	2	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. broomii	intermediate	1	1	Europe	human	(Levett *et al*., 2006)

Species	Virulence	Strain (N)	Genomes (N)	Geographic origin	Source of isolation	Reference
L. congkakensis	probable saprophytic	4	3	Asia	environmental	(Vincent *et al*., 2019)
L. dzianensis	probable pathogenic	7	6	Africa - Asia	environmental	(Vincent *et al*., 2019)
L. dzoumogneensis	probable intermediate	2	1	Africa	environmental	(Vincent *et al*., 2019)
L. ellinghausenii	probable saprophytic	1	1	Asia	environmental	(Vincent *et al*., 2019)
L. elllisii	pathogenic	2	2	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. fainei	intermediate	1	1	Oceania	porcine	(Perolat *et al*., 1998)
L. fletcheri	probable intermediate	1	1	Asia	environmental	(Vincent *et al*., 2019)
L. fluminis	probable intermediate	1	1	Asia	environmental	(Vincent *et al*., 2019)
L. gomenensis	probable pathogenic	5	4	Oceania	environmental	(Vincent *et al*., 2019)
L. haakeii	intermediate	2	2	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. harrisiae	probable saprophytic	2	2	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. hartskeerlii	intermediate	2	2	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. idonii	saprophytic	2	1	Asia	environmental	(Saito *et al*., 2013)
L. ilyithenensis	probable saprophytic	1	1	Africa	environmental	(Vincent *et al*., 2019)
L. inadai	intermediate	8	2	Africa - Asia - North America – Oceania - South America	bandicoot - human - mongoose - opossum - rodent	(Matthias *et al*., 2008)

Species	Virulence	Strain (N)	Genomes (N)	Geographic origin	Source of isolation	Reference
L. interrogans	pathogenic	845	365	Africa - Asia - Europe - North America - Oceania - South America	amphibians - bandicoot - bat - bovine -canine - environmental - equine - hedgehog - human - marine mammal - opossum - ovine - porcine – shrew	(Faine and Stallman, 1982; Yasuda *et al*.,1987)
L. jelokensis	probable saprophytic	3	2	Asia	environmental	(Vincent *et al*., 2019)
L. johnsonii	probable intermediate	1	1	Asia	environmental	(Vincent *et al*., 2019)
L. kanakyensis	probable saprophytic	4	3	Oceania	environmental	(Vincent *et al*., 2019)
L. kemamanensis	probable saprophytic	2	1	Asia	environmental	(Vincent *et al*., 2019)
L. kirschneri	pathogenic	86	33	Africa - Asia - Europe - North America - South America	bat - bovine - canine - environmental - equine - hedgehog - human - porcine - rodent - shrew	(Ramadass *et al*., 1992)
L. kmetyi	pathogenic	8	7	Asia - Oceania	environmental	(Slack *et al*., 2009; T hibeaux *et al*., 2018a)
L. kobayashii	probable saprophytic	1	1	Asia	environmental	(Vincent *et al*., 2019)
L. koniamboensis	probable intermediate	2	1	Oceania	environmental	(Vincent *et al*., 2019)
L. langatensis	probable intermediate	2	2	Asia	environmental	(Vincent *et al*., 2019)
L. levettii	probable saprophytic	14	14	Africa - Asia - Oceania	environmental	(Thibeaux *et al*., 2018a)

Species	Virulence	Strain (N)	Genomes (N)	Geographic origin	Source of isolation	Reference
L. licerasiae	intermediate	6	4	Africa - South America	bat - environmental - human - opossum	(Matthias *et al*., 2008)
L. mayottensis	pathogenic	6	6	Africa	environmental - human - tenrec	(Bourhy *et al*., 2014)
L. meyeri	saprophytic	13	11	Africa - Asia - Europe - North America - Oceania	bandicoot - environmental - human - rodent	(Thibeaux *et al*., 2018a; Yasuda *et al*., 1987)
L. montravelensis	probable saprophytic	3	2	Oceania	environmental	(Vincent *et al*., 2019)
L. mtsangambouensis	probable saprophytic	2	1	Africa	environmental	(Vincent *et al*., 2019)
L. neocaledonica	intermediate	1	1	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. noguchii	pathogenic	53	24	Asia - North America - South America	amphibians - armadillo - bovine - canine - human - mongoose - nutria - opossum - porcine - rodent - weasel	(Yasuda *et al*., 1987)
L. noumeaensis	probable saprophytic	2	1	Oceania	environmental	(Vincent *et al*., 2019)
L. ognonensis	probable saprophytic	1	1	Europe	environmental	(Vincent *et al*., 2019)
L. perdikensis	probable saprophytic	2	1	Asia	environmental	(Vincent *et al*., 2019)
L. perolatii	intermediate	2	2	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. putramalaysiae	probable pathogenic	2	2	Asia	environmental	(Vincent *et al*., 2019)
L. ryugenii	probable saprophytic	1	1	Asia	environmental	(Vincent *et al*., 2019)

Species	Virulence	Strain (N)	Genomes (N)	Geographic origin	Source of isolation	Reference
L. saintgironsiae	intermediate	1	1	Oceania	environmental	(Thibeaux *et al*., 2018a)
L. santarosai	pathogenic	109	40	Africa - Asia - North America - South America	bovine - canine - caprine - capybara - environmental - human - opossum - porcine - raccoon - rodent	(Yasuda *et al*., 1987)
L. sarikeiensis	probable intermediate	2	1	Asia	environmental	(Vincent *et al*., 2019)
L. selangorensis	probable intermediate	3	3	Asia	environmental	(Vincent *et al*., 2019)
L. semungkisensis	probable intermediate	1	1	Asia	environmental	(Vincent *et al*., 2019)
L. stimsonii	probable pathogenic	2	2	North America	environmental	(Casanovas-Massana et al., 2019)
L. terpstrae	Unknown	1	1	Asia	Unknown	(Smythe *et al*., 2013)
L. tipperaryensis	probable pathogenic	1	1	Europe	Shrew	(Vincent *et al*., 2019)
L. vanthielii	saprophytic	2	2	Europe	environmental	(Smythe *et al*., 2013)
L. venezuelensis	intermediate	3	3	South America	bovine - human - rodent	(Puche *et al*., 2018)
L. weilii	pathogenic	39	20	Asia - Europe - North America - Oceani	amphibians - bovine - canine - environmental - equine - human	(Yasuda *et al*., 1987)
L. wolbachii	saprophytic	1	1	North America	environmental	(Yasuda *et al*., 1987)
L. wolffii	intermediate	7	7	Asia - Oceania	environmental - human	(Slack *et al*., 2008; Thibeaux *et al*., 2018a)

Species	Virulence	Strain (N)	Genomes (N)	Geographic origin	Source of isolation	Reference
L. yanagawae	Unknown	2	2	Oceania - South America	environmental	(Smythe *et al*., 2013)
L. yasudae	probable pathogenic	2	2	South America	environmental	(Casanovas-Massana *et al*., 2019)
Leptospira sp.	-	14	3	North America Journal Pre-proof Asia - Oceania Oceania - South America South America	bovine - environmental - human - opossum - rodent	-
TOTAL		**1563**	**704**			

Adapted from Caimi *& Ruybal; 2020 [28].* The total number of strains and genomes are shown based on the available information on the databases since some strains are present more than once, and some species have limited number of available sequenced genomes.

This pathogenicity-based classification has been mostly done by genetic analysis of 16S rRNA sequence and complemented by DNA-DNA hybridization against reference strains [29, 30]. Recently, Vincent *et al.* [25] have suggested a new approach for *Leptospira* classification, where a systematic classification scheme is proposed for *Leptospira* species based on comparison of average nucleotide identity (ANI), genome diversity, pan-genome and core gene sets. This new approach showed the existence of two clades and 4 subclades, allowing the identification of genome features, genes and domains that are important for each subclade. It is clear that the *Leptospira* field is going through some big changes, especially now that more genetic tools are becoming available. Based on available information, this work refers to species and serovar following the previous classification present in the literature.

At of the subspecies level, leptospires are classified into serovars accordingly to differences on the carbohydrate structures of their lipopolysaccharide (LPS) [31]. In 1954, Wolff and Broom suggested the use of the term serotype for the basic taxonomic unit of a serological classification, and that closely related serotypes could be clustered into serogroups for convenience [31]. Today, nearly 300 different serovars have been identified and the list of serovars is being continuously updated [28]. Serological classification is mostly performed using a microscopic agglutination test (MAT), a test that was developed in 1918 by Martin and Pettit [32] and since then, this technique remained as the gold standard method for leptospirosis diagnosis, with little change over the years [33].

Like many other tests, MAT presents many technical limitations. It requires maintenance of live leptospiral cultures of the reference strains, it is hard to standardize because of its dependence on the operator, and it frequently presents cross-reactions between serogroups, where depending on the conditions, such as clinical phase, it is considered inappropriate to detect infecting serovar [34, 35]. Yet, the MAT test strategy on detecting regional circulating serovars and collecting prevalence data continues to be highly applicable [36-38]. This understanding of geographic factors and leptospiral serovar prevalence constitutes the basis for the design and decision making on control strategies such as vaccination.

Pathogenesis and virulence factors

The mechanisms by which leptospires cause disease are still not well understood, but due to the latest developments an application of genetic tools in the study of *Leptospira* have led to advances on understanding its pathogenicity and virulence factors, by the assessment of mutants in animal models [39]. Some of the essential virulence factors for *Leptospira* have been identified, but most still do not have a clearly and well defined function.

Improvements have also been made in the *in vitro* characterization of interaction between leptospires and host structures, including soluble mediators of complement resistance (factor H, C4b-binding protein), extracellular matrix proteins (such as laminin, elastin, fibronectin, collagens), and proteins related to hemostasis (fibrinogen, plasmin), although none of these *in vitro* findings has

been translated to the host animal [39]. These interactions must play important roles during leptospiral infection, for example: binding to host cellular structures can allow colonization; preventing blood from clotting contributes to hemorrhage, while the interaction of leptospiral outer membrane proteins and extracellular factors with complement mediators may contribute to bacterial survival while in the blood.

Some leptospiral proteins and genes have been shown to not be considered virulence factors, as mutants with deletions of such genes have proven virulent [29]. Some of these proteins include: the major outer membrane lipoprotein LipL32 [40, 41], the leptospiral protein LipL41 was shown to not be required for acute infection of hamsters [42], the Len family proteins B and E, metalloprotease, the lipoprotein LigC, chemotaxis proteins CheB and CheX, and the Fur family regulator PerR [43]. Interestingly, the lipoprotein LigB, a leptospiral protein that belongs to the superfamily of bacterial immunoglobulin-like (Big) repeat domain proteins, a superfamily that includes virulence factors from enteropathogenic *Escherichia coli*, *Yersinia pseudotuberculo*sis and *Bordetella* spp. [44], was pointed to as a non-virulence factor for *Leptospira* [45].

On the other hand, many genes and proteins have been shown to be related with virulence, thus being necessary for survival and colonization. Not surprisingly, some of the LPS biosynthesis genes are necessary for viability and virulence [43, 46, 47]. Another common key virulence factor is motility, and for *Leptospira* it has been shown to be a key virulence factor as early as 1964 by

Faine & Vanderhoeden [48]. With later development of new technologies that allow easier genetic manipulation, confirmation about motility came with mutation on FliY, a flagellar switch protein, attenuated the virulence in guinea pigs [49]; the inactivation of FliM protein, the flagellar motor switch protein, attenuating the virulence of *L. interrogans* serogroup Australis strain 702 in hamsters [50]. The mutation on FlaA2 and FcpA, a flagellar structural and a flagellar sheath protein, also led to attenuated virulence [51, 52], demonstrating clearly that leptospiral motility is essential for virulence.

Iron acquisition is fundamentally important for so many pathogenic bacteria as it is for *Leptospira*, where the inactivation of the *hemO* gene resulted in attenuation of acute disease in hamsters, though the mutant retained the ability to colonize hamster kidneys [53, 54]. Genes involved with oxidative stress response were are also shown to be necessary for virulence, as demonstrated by mutation of leptospiral catalase protein, KatE, from pathogenic leptospiral strains [55].

Mce, a cell entry protein, although it is not essential but it may have a virulence-associated function [56]. Leptospiral collagenase, ColA, can hydrolyze several host collagens *in vitro*, but its inactivation resulted in only a minor reduction of virulence, suggesting a minor but non-essential role in pathogenesis [57].

There are also some virulence genes of unknown or undefined function, like Loa22, a putative lipoprotein with a predicted OmpA domain has been shown to be essential for virulence but whose function is either unknown or poorly

understood [58]. The LruA surface protein has also been shown to be a virulence factor, which plays a major role in provoking leptospiral uveitis in horses via an autoimmune mechanism, but the whole function of LruA remains unclear [59]. Other supposed virulence genes have been reported, but none have had its function studied [46, 60, 61].

Several mutants in genes that were expected to be involved in virulence, ended up not affecting the virulence at all, demonstrating that leptospires display a high level of functional redundancy; where some of these genes' products might be compensated by others when deleted experimentally. Genes of unknown function are over-represented in pathogenic *Leptospira* spp., which suggests that leptospires may have unique, but yet uncharacterized virulence mechanisms. The study of these supposed virulence factors as well as the discovery and study of new genes is fundamental for the advance on the leptospirosis field and the understanding of leptospiral infection mechanism.

Epidemiology

Leptospirosis is responsible for about 1 million cases and 60,000 fatalities annually worldwide, where it mostly affects rural or suburban populations in tropical and subtropical regions where longer survival of leptospires in the environment is favored by warm and humid conditions [4]. Case-fatality rates range from <5% to 30%. Most tropical countries are also developing countries, where normally the opportunities for exposure of the human population to

infected animals, whether livestock, domestic pets, or wild or feral animals, is greater.

Transmission of *Leptospira* spp. among animals and humans can occur through contact with abrasions, cuts in the skin or via the conjunctiva with some risk factors like mud, water or food that is contaminated with urine from an infected host, and in special cases some strains rely on a host-to-host transmission [62].

The reported incidence of leptospirosis is mostly based on the availability of laboratory diagnosis and the clinical index of suspicion as much as the incidence of the disease. For example, the real incidence of leptospirosis is not well documented in the Asia Pacific region, predominantly in developing countries, and therefore the leptospirosis burdens are often underestimated [63, 64]. Additionally, leptospirosis has emerged as a health threat in new settings due the influence of globalization and climate. Extreme weather events and natural disasters are now recognized to precipitate epidemics [65]. Lastly, the uncontrolled growth of urban slums worldwide has created perfect conditions for rat borne transmission, therefore favoring leptospiral transmission [66-69].

These factors highlight the need for better and easier methods for leptospirosis diagnosis, methods that will not depend and rely on specific laboratories and personal, and that could be used broadly, while lowering test costs and increasing reports.

Leptospirosis in horses

Like humans, horses are considered accidental hosts, because they are not typically carriers of the disease but are susceptible to leptospiral infections under certain circumstances, like direct exposure to a reservoir host. Equine leptospirosis is most commonly associated with diseases of the placenta and fetus, the kidneys and the eyes [70]. Leptospiral infections in pregnant mares may result in abortion [10]. Acute renal failure in younger horses [14] and equine recurrent uveitis (ERU) in adult horses [8, 9] are also well documented clinical syndromes of equine leptospirosis. Although abortions, neonatal disease and acute renal failure are normally caused by subacute infections, ERU can affect horses months or years after the initial *Leptospira* infection [12, 70]. Mares that abort due to leptospiral infection might have no additional clinical signs at the time of abortion, but these mares can shed the *Leptospira* spp. in the urine for several weeks. Because of the non-specific clinical signs associated with leptospirosis, the disease in horses may occur more frequently than is diagnosed, and exposure to *Leptospira* spp. may be more prevalent than was previously thought. The understanding of leptospiral infection in horses and the complications caused by these infections is really important, as well as the understanding of leptospiral serovars that might pose a threat, not just for horses, but also for other hosts that might be exposed.

Laboratory diagnosis

In humans, leptospirosis generally presents itself with common flu-like symptoms, presenting a febrile stage during the acute phase infection, which

makes the early clinical diagnosis more complicated [71]. This early diagnosis is crucial for proper treatment and to alleviate the disease severity, where the wrong treatment can prime a systemically spread of leptospires, and it might lead to a more serious and advanced stage. Consequently, leptospirosis diagnosis is largely dependent on available and specific laboratory tests.

Laboratory diagnosis of leptospirosis can made by a combination of antibodies or antigens testing methods, or by isolation of leptospires in cultures [71, 72] or by targeting leptospiral DNA by polymerase chain reaction (PCR) [73, 74]. Although there are many methods for detecting a leptospiral infection, the gold standard test is the MAT, that detects agglutinating antibodies in the horse serum against the bacterium that are more evident on a later phase, also named “immune phase” or convalescent phase [32]. However, the ability of convalescent-phase MAT titers to predict even the infecting serogroup may be as low as 40% [1]. The MAT test can serve as a basis to detect seroprevalence rates, to provide information on exposure rates and suspected infecting serogroups in the geographic region being studied, but it does not present any critical information about the carrier or shedding status of the host. This test presents a high diagnostic specificity, but on the other hand, it offers a relatively low sensitivity [34].

Other methods have been shown to be suitable for diagnosis depending of the disease clinical phase. The polymerase chain reaction (PCR) targeting leptospiral genes was shown to be a useful molecular detection tool for rapid

qualitative diagnosis in the earliest stage [73-78]. Another strategy that can help to confirm leptospiral infection during acute phase is by blood culture isolation. However, this method is very time consuming, it requires specialized media and equipment, it can take months for a serovar specific result and in some cases the contamination of the sample can make isolation almost impossible [15].

Other tests for leptospirosis diagnosis have been developed over the years including complement fixation testing [79], flow cytometry [80], other immune assays like lateral flow tests [81, 82], proteomic array [83] and dual path platform [84]. Many enzyme-linked immunosorbent assays (ELISAs) using recombinant proteins have also been shown to fit the diagnosis criteria for an accurate and valuable test, like *Leptospira* immunoglobulin-like proteins (LigA and LigB) [85-90], and other proteins: LipL32 [88, 91-93], Loa22 [88, 93], LipL21 [88, 93, 94], OmpL1 [94], LipL41 [93]. The ELISA has the advantage of its simplicity, short-test performance and sensitized microwell plates that can be kept for months before use, the ability of running multiple samples and/or multiple antigens in only one plate [95]. New discoveries are made and proteins are been tested constantly in order to improve leptospirosis diagnosis and help to formulate protective vaccines [83, 96, 97].

In summary, the field of leptospirosis has undergone many great advances over the last few decades, but there are many gaps in our knowledge about these bacteria that need attention. The study of leptospiral features and how these, proteins and genes, are involved during the stages of infection are still vague.

There is a need for identification and study of leptospiral antigens, for the development of an easier and more accurate diagnostic test, and hopefully in the near feature, the development of a multi-serovar vaccine, that could be beneficial for both, humans and animals.

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CHAPTER TWO

LEPTOSPIROSIS IN HORSES: EXPERIMENTAL INFECTION OF *L. INTERROGANS* SEROVAR BRATISLAVA IN HORSES, IMMUNOLOGICAL ANALYSIS AND A BETTER UNDERSTANDING OF SEROLOGICAL RESPONSE

Introduction

Leptospirosis is considered to be the most wide spread zoonosis in the world, infecting humans and many animal species [1, 2]. The disease is caused by pathogenic *Leptospira* spirochetes, which can induce a wide range of clinical manifestations depending on the serovar and host. In humans, the symptoms can range from a mild flu-like sickness to severe Weil's disease characterized by multi-organ failure, and in some cases leading to death [3, 4]. In animals, especially in horses, some serovars are associated with many clinical syndromes, including pyrexia [5], uveitis [6, 7], abortion [8], recurrent uveitis [9] and renal failure [10].

The microscopic agglutination test (MAT) is currently the "gold standard" test used for detecting leptospiral antibodies in which serum from suspected hosts reacts with live antigen suspensions of different *Leptospira* serovars. MAT was developed soon after the first isolation of leptospires, more than 100 years ago, and serological data derived from the MAT started to be routinely used to infer the infecting leptospiral serovar. At that time just few serovars were known,

which made it easy to include all those serovars known to occur within a region in the antigen panel and to interpret the results of serologic testing as being serovar specific [11, 12]. Nowadays, more than 300 serovars are reported [13], and it became clearer that serovar specificity was an erroneous concept [14]. MAT test can serve as basis to determine the seroprevalence rate [15-17], to provide information on exposure rates and identify suspected infecting serogroups in the geographic region being studied [14, 18-20], but it does not provide critical information about the carrier or shedding status of the host. Moreover, because antibodies to *Leptospira* may take several weeks to become detectable by MAT, the use of such test is more appropriate during the convalescent-phase [21], a later stage that happens normally 2 to 3 weeks after the acute stage where the first symptoms are detected [22]. This results in a less precise and possibly misleading identification of infecting serovar during the acute phase. Although MAT usually presents high specificity, it is a labor-intensive method and not highly sensitive, having high cross reactivity between different serovars. Therefore, the MAT results should be only used for an estimate on the infecting serovar [21], but not for confirming the infecting serovar. This lack of sensitivity, combined with a high cross-reactivity among serovars and the unknown clinical phase stage, can be a problem during the diagnosis of leptospirosis, since agglutination antibodies may not be seen for a couple of weeks following infection in some cases [23].

To strengthen diagnostic testing, one strategy could be the use of more than one method to detect and identify the strain (or the serovar) that is causing the

infection, increasing the specificity and sensitivity [21]. Isolation and Whole Genome Sequencing (WGS) could be a better option for those cases where there is a clear correlation between the high levels of antibodies and the presence of clinical signs such as abortion, renal failure or in uveitis cases that have more modest titers due to both chronic and localized infection. Although, the difficulty of culturing samples can limit the ability to identify the strain/serovar that is causing the disease. However, the use of other methods like PCR [24], microarrays [25] and microsphere immunoassay [26] can help to increase the efficacy of diagnostic testing. There remains a need for a better, faster and safer serological test for leptospirosis, also a test that could be broadly used and not require a very well trained personal, making the test availability a problem, lowering the reports as well.

The *L. interrogans* serovar Bratislava is thought to be an equine-adapted serovar [27-32], especially in North America, where random serologic surveys based on MAT indicate that as many as 52% of adult horses have agglutination antibodies against this serovar [30]. Although serological detection of this serovar is frequently described in horses by the means of MAT reports/results, its role in the clinical presentation of the disease has yet not been proven by an experimental challenge. On the other hand, the role of the serovar Bratislava in reproductive disorders has been clearly demonstrated in pigs; it has been isolated from the kidney and uterus in repeat-breeding sows [33] and the genome sequence has been made available in most of these cases. Serological identification of this serovar in clinical cases of leptospirosis has been reported

over the years. In Brazil, Pinna *et al.*[34] proposed that higher titers against this serovar were correlated with horses that presented reproductive problems, but no isolation nor genetic confirmation was made. Baverud *et al.* [17] found from an owner/trainer survey in Sweden, that there was no significant association between clinical signs, disease and positive titres against *L. interrogans* serovar Bratislava in horses except for more respiratory problems and fatigue reported in the seropositive horses. It was also reported that seroprevalence to *L. interrogans* serovar Bratislava increased with age, which in itself could explain the owner reports on increased respiratory disease and fatigue in the studied horses. Around 20% (65 out of 335 animals) of the seropositive horses for *L. interrogans* serovar Bratislava were healthy horses, and of these 24.6% (16 out of 65 animals) had higher titers (≥1:200), a higher proportion than the non-healthy animals (16.6%) against the same serovar. Park *et al.* [35], in 1992, also reported the prevalence of seroreactivity for *L. interrogans* serovar Bratislava among horses in Ohio. One horse showed colic as the predominant clinical sign, and serum analyzed by MAT showed high titers against *L. interrogans* serovar Bratislava (≥1:6400), which were higher than titers to other serovars. But interestingly, five days later the agglutination antibody titres against *L. interrogans* serovar Pomona increased from 1:800 to ≥1:6400, while titres against *L. interrogans* serovar Bratislava decreased to 1:400. The dynamics of the agglutination titers indicates that the infection was probably caused by *L. interrogans* serovar Pomona and not by serovar Bratislava, and in this case, a follow-up sample clearly showed the misleading information provided by MAT

results during the first sampling. This inconsistency and cross-reactivity among serovars over time can complicate the diagnosis of leptospirosis, affect treatment, harm the patient and mislead the identification of the infecting serovar, which could lead to an erroneous decision when planning for a vaccine approach. Moreover, these results raise questions about the pathogenicity of *L. interrogans* serovar Bratislava for horses which were investigated in the present work.

In this study, the experimental challenge was performed in horses with *L. interrogans* serovar Bratislava - strain PigK151 and documented serology, PCR analysis, clinical signs, biochemical, hematological and histological observations following infection. The findings from this work suggest that *L. interrogans* serovar Bratislava is possibly not a host-adapted serovar for horses, and the cross-reactivity, which led to the previous postulate, gains new important insights, helping to improve the knowledge on the leptospirosis field.

Methods

The protocol of this study was approved by the Institutional Animal Care and Use Committee at Cornell University. All work was conducted in compliance with regulations, policies, and principles of the Animal Welfare Act; the Public Health Service for Policy on Humane Care and Use of Laboratory Animals used in testing, research, and training; the NIH Guide for the Care and Use of Laboratory Animals; and the New York State Department of Public Health regulations. Ten mixed breed, female horses (7-8 months old) were tested using the microscopic agglutination test (MAT). All the animals were seronegative (titers <50 at day -30 and -1 prior to inoculation) by MAT and negative by PCR targeting the gene *lipL32* on *L. interrogans* serovar Bratislava on plasma and urine.

Bacterial strain

The *L. interrogans* serovar Bratislava - strain PigK151, obtained from the National Animal Disease Center, and was used for this study [36]. In order to isolate low-passage cultures of leptospires, hamsters were experimentally infected with a sub-lethal dose of *L. interrogans* serovar Bratislava - strain PigK151. Infected hamster tissues were harvested aseptically and homogenized with sterile phosphate-buffered saline. The isolates were maintained on Ellinghausen, McCullough, Johnson, and Harris (EMJH)[33] medium supplemented with 1% rabbit serum (Rockland Immunochemicals, Gilbertsville, PA) and 100 µg/ml 5-fluorouracil (5-FU), at 30°C and later frozen in liquid nitrogen. Several aliquots from previous isolated cultures (frozen vials)

were cultured as previously mentioned and growth was monitored using dark-field microscopy. Prior to challenge, *Leptospira* cells were centrifuged at 1000 rpm for 45 min at RT and resuspended in phosphate saline buffer (PBS, pH 7.4) to reach final concentrations of 1 x 10^9 cells/mL and 5 x 10^8 cells/mL. Cell viability was analyzed by dark field microscopy and quantified by a direct count in a Petroff-Hauser counting chamber.

PCR analysis

A pair of primers was created to allow the detection of the major outer membrane protein LipL32, which has been used before in diagnostic tests [37-40]. The *LipL32* sequence was extracted from the database at the Pathosystems Resource Integration Center [41] (PATRIC, www.patricbrc.org - fig|338215.3.peg.1694). The primer sequences used were: LipL32Brat-Fp (5'-CCA-TGG-atg-aaa-aaa-ctt-tcg-att-ttg-gc-3') encoding a "NcoI" digestion site at the 5' end and used as the forward primer; and LipL32Brat-Rp (5'-CTC-GAG-tta-ctt-agt-cgc-gtc-aga-ag-3') encoding a "XhoI" digestion site at the 5' end and used as the reverse primer. Amplification of the 819bp sequence coding *LipL32* was done using DreamTaq™ Hot Start Green PCR Master Mix (Thermo Fisher Scientific) as follows: 95°C for 3 min, 35 cycles of 95°C for 30 s followed by 50 °C for 1 min and 72 °C for 1 min, and then 72 °C for 10 min to complete the amplification. Primers were validated using DNA isolated from bacterial cultures of *L. interrogans* serovar Bratislava, *L. interrogans* serovar Pomona, *L. interrogans* serovar Hardjo, *L. interrogans* serovar Lai, *L. interrogans* serovar

Canicola, *L. interrogans* serovar Grippotyphosa and *L. interrogans* serovar Autumnalis (data not shown).

Animal model and infection study

We stipulated that colonization and/or clinical complications would be present in 80% of the challenged animals and in less than 0.5% of the control group. With power of 80%, two-sided significance level (1-α) of 95% and sampling ratio of 2:3 for control: challenged, and based on the Fleis [42] method of sample size calculation, the sample size obtained was 6 subjects for the challenge group and 4 in the control group. Therefore, ten seronegative foals were assigned randomly to the 2 respective groups. Rectal temperature, blood and urine were collected prior to the infection. Due to the lack of a well stablish equine model for leptospirosis, the routes of inoculation were based on a previous study [5]. As part of the negative control group, 4 horses received 500 µL of PBS by topical ocular injection and 1 mL of PBS via IP injection. For the challenged group, six horses received 500 µL of 1 x 10^9 cells/mL by topical ocular injection and 1 mL of 5 x 10^8 cells/mL via IP injection, corresponding to a total dose of 10^9 cells divided equally between the two sites. All horses were maintained separately in equine isolation facilities free from contact with other horses and strict biosecurity was maintained between the horses. Subsequent to the challenge, temperature and physical examinations were performed twice daily for 9 days and then weekly.

After sedation with detomidine hydrochloride (10 µg/kg, IV - Dormosedan, Zoetis, Inc., Kalamazoo, MI) 5-10 mL blood samples were collected aseptically by jugular venipuncture on days 3, 5 and 7 and weekly until the end of the experiment. At any sign of fever or beginning on day 3 post challenge and every other day, an automated complete blood count (CBC), differential and chemistry panel (evaluation of liver, kidney and muscle damage) were performed. Around 50-200 mL of urine was collected on day 3, 5 and 7 and weekly until the end of the experiment. DNA was extracted from plasma and urine samples using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's recommendations and further tested by PCR using *LipL32* primers as described.

Sera from all the animals were tested by MAT as described previously [43]. Briefly, serial 2-fold dilutions of the sera, starting with a dilution of 1:10, were mixed with an equal volume of viable *Leptospira* strains (*L. interrogans* serovar Pomona, *L. interrogans* serovar Hardjo, *L. interrogans* serovar Icterohaemorrhagiae, *L*. *kirschneri* serovar Grippotyphosa, *L. interrogans* serovar Canicola, *L. interrogans* serovar Autumnalis and *L. interrogans* serovar Bratislava) in a 96-well microtiter plate. After incubation at 30°C for 2 h, the samples were examined for agglutination by dark field microscopy. Titres are the reciprocal of the highest serum dilution showing 50% agglutination of the leptospiral cells in the suspension.

At 4 weeks after inoculation the horses, were euthanized with an overdose of pentobarbital (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI) given intravenously (90 mg/kg, IV). Representative sections from liver, kidney, urinary bladder, uterine body and pineal gland were fixed by immersion in 10% neutral buffered formalin. Eyes were fixed for 24 hours in Bouin's fixative and then transferred to 70% ethanol. The tissues were embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (H&E) using standard histologic techniques. The sections were examined by a board certified veterinary pathologist (SPM), who was blinded to the treatment groups. Culture samples of blood and urine were done as previously described [5]. Briefly, 1 mL of blood and urine, or 0.5-1 mL of undiluted vitreous, were added to both liquid and semisolid EMJH medium with 5-FU and maintained at 30°C for up to 12 weeks. The presence of *L. interrogans* serovar Bratislava was monitored with dark field microscopy weekly. The presence of *L. interrogans* serovar Bratislava was monitored with dark field microscopy weekly. Also, tissue samples from liver, kidney and reproductive tract were prepared as described elsewhere [28, 44] with a few modifications. Briefly, about 1g of each tissue collected aseptically was disrupted by use of syringe and collected in tubes with sterile phosphate buffered solution (PBS). The tubes were vortexed and later centrifuged for 10 minutes at 3,000 rpm. The presence of Leptospires was checked by dark field microscopy from the supernatant and three aliquots from each supernatant were inoculated into supplemented semisolid EMJH medium, maintained at 30 °C for up to 12 weeks and the presence of *L. interrogans* serovar Bratislava was

monitored by dark field microscopy weekly. DNA was extracted from all tissue samples using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's recommendations and further tested by PCR using *LipL32* primers as described.

Statistical analysis

Statistical analysis was performed as described in each figure legend using Origin(Pro), Version 2018 software (OriginLab Corporation, Northampton, MA, USA). A two-tailed t-test was used for pairwise comparisons between groups. MAT Geometric means for the seven serovars were compared by analysis of variance (ANOVA), and Tukey test was used to find which specific mean was significantly different between serovars at each sampling date. All data was considered significant when $P<0.05$.

Results

Clinical signs

Rectal temperature remained within the normal horse range (37.2 - 38.6 °C) for the first 7 days following inoculation as shown in Figure 2.1. Temperature was taken in the mornings (am) and afternoons (pm) from days 1 to 9, then once daily.

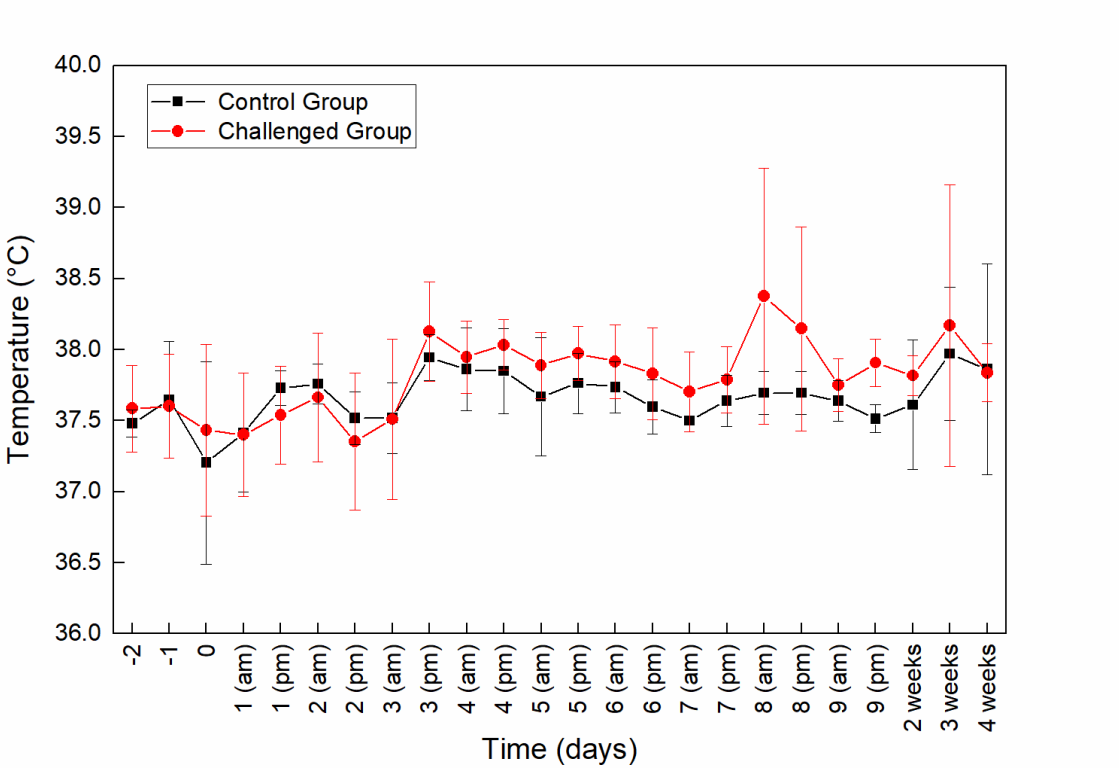

Figure 2.4: Daily body temperature.

Two challenged horses developed fever (once per subject) at different time points of the study: on days 8, for a 24-36 hour period, and in weeks 3 respectively. Blood and urine collected at the time of fever were analyzed by PCR and results were negative for the detection of the gene *lipL32*. The temperature returned to the normal range within the same day or a day later for these animals. No significant difference in the temperature (P = 0.3) was found between groups. It was not possible to detect *Leptospira* in either urine or blood, and therefore it is believed that these episodes of fever could be due to factors other than the challenge itself. Despite these three observations, all animals were bright and responsive during the experiment and no other abnormal clinical or physical signs were noticed.

Clinical Pathology

Complete blood count (CBC) and serum biochemical profiles are shown in Figure 2.2. The two-tailed t-test was used to test for statistically significant differences ($P<0.05$) between the control and challenged groups among the tested parameters. The challenged group had a significantly higher WBC ($P = 0.02$) and lymphocytes ($P = 0.0001$) than the control group. The iron content in the blood of the challenged group was significantly lower ($P = 0.04$) than the control group, with the concentration range dipping below the normal reference range (95-217 µg/dL). These differences are probably due to the challenge, where the inoculation of *L. interrogans* serovar Bratislava triggered an inflammatory response and the horses' immune system, increasing the number of innate and adaptive immune effector cells that helped to clear the bacteria. It

was also noted that the challenged group presented a significantly higher concentration of total bilirubin (P = 0.001) even though the group concentration range for bilirubin stayed within the reference interval limits (0.5 - 2.1 mg/dL), despite the bilirubin level of 3.4 mg/dL experienced by horse 8 during its fever occurrence on day 21. GLDH levels were significantly higher (P = 0.03) for the challenged group when compared with the controlled group but all horses remained within the normal reference range. The context of clinical chemistries at the individual level and temperature are available in the Supplementary Material S1.

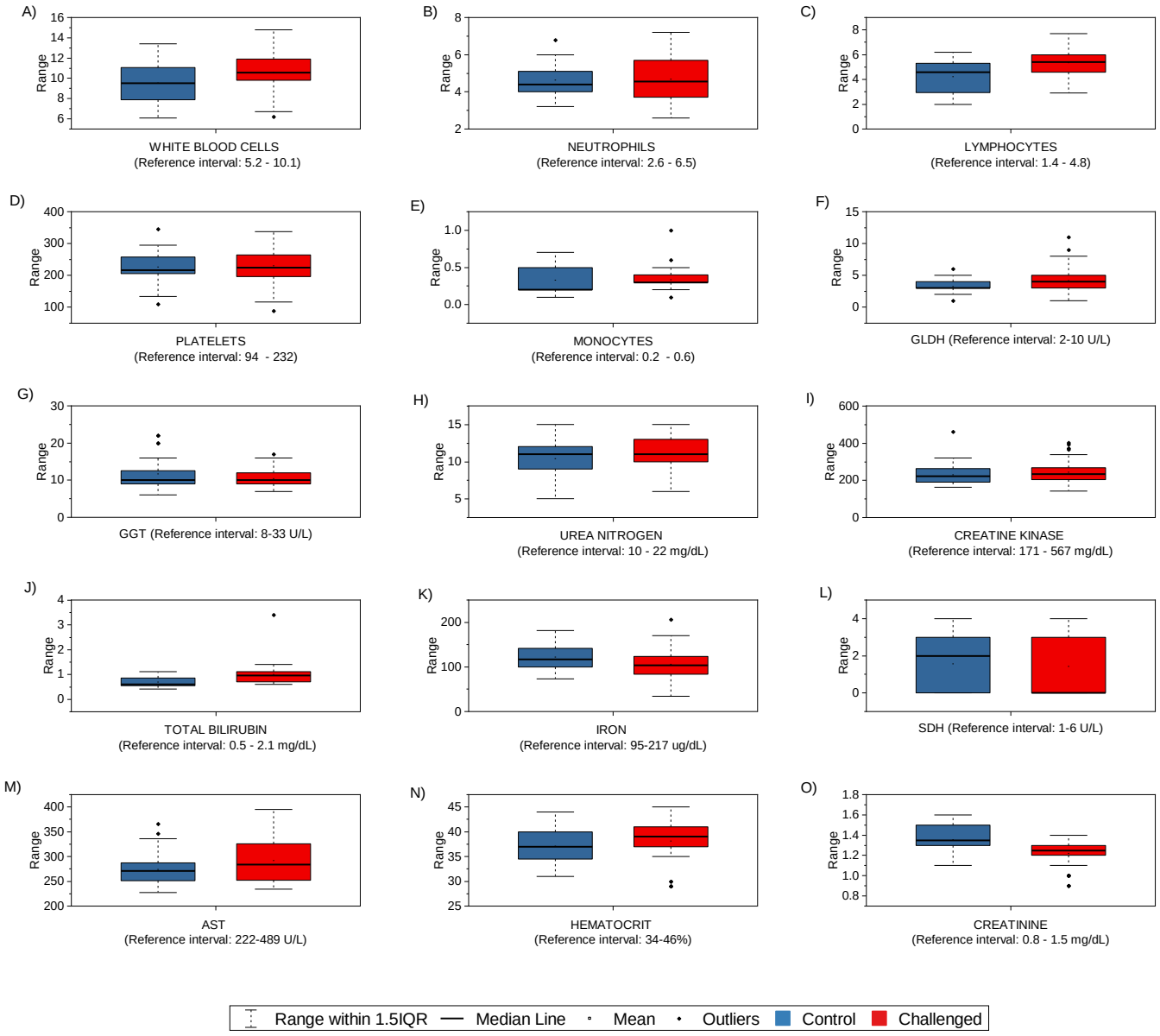

Figure 2. 5: Complete blood count automated (CBCA), differential, and chemistry panel results.

Bacteriology and PCR analysis

Although a humoral response was detected as early as 3 days, suggesting a possible infection, no Leptospires were detected by dark field examination of cultures prepared from aqueous humor, urine, kidney, liver, or urinary bladder from any horse, nor by PCR from the same tissues, including samples from plasma or urine collected throughout the study.

Histopathology

No evidence of leptospirosis was found in either the control or challenged groups as can be seen in Figure 2.3. Clinically irrelevant mild lymphocytic conjunctivitis was noted in 4/4 control horses and 3/6 challenged horses. Mild lymphocytic cystitis occurred in 2/4 control horses and 3/6 of those challenged with *L. interrogans* serovar Bratislava. No hepatitis or nephritis was present but two challenged horses had very mild lymphocytic endometritis (Kenney-Doig grades 1 and 2A). One control horse had a parasitic granuloma in the liver. Extra information and pictures are provided in Supplementary Material S2.

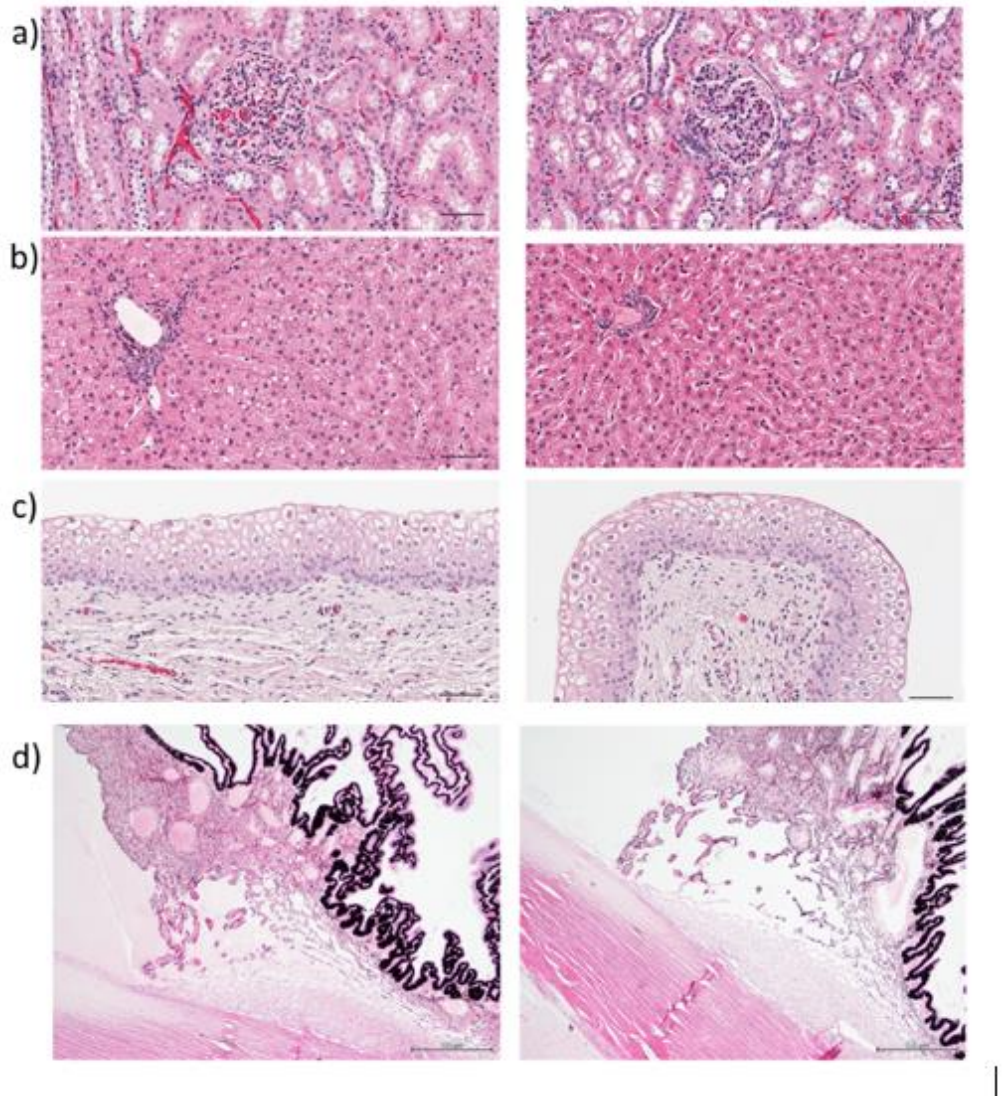

Figure 2.6: Histopathological photomicrographs. Left column is from a horse from control group while the right column is from a challenged horse. a) kidney; b) liver; c) urinary bladder and d) iridociliary angle of the eye. No significant lesions were present in either group.

Serology

All foals in the challenged group developed an agglutination titer against *L. interrogans* serovar Bratislava and geometric means as shown in Figure 2.4.

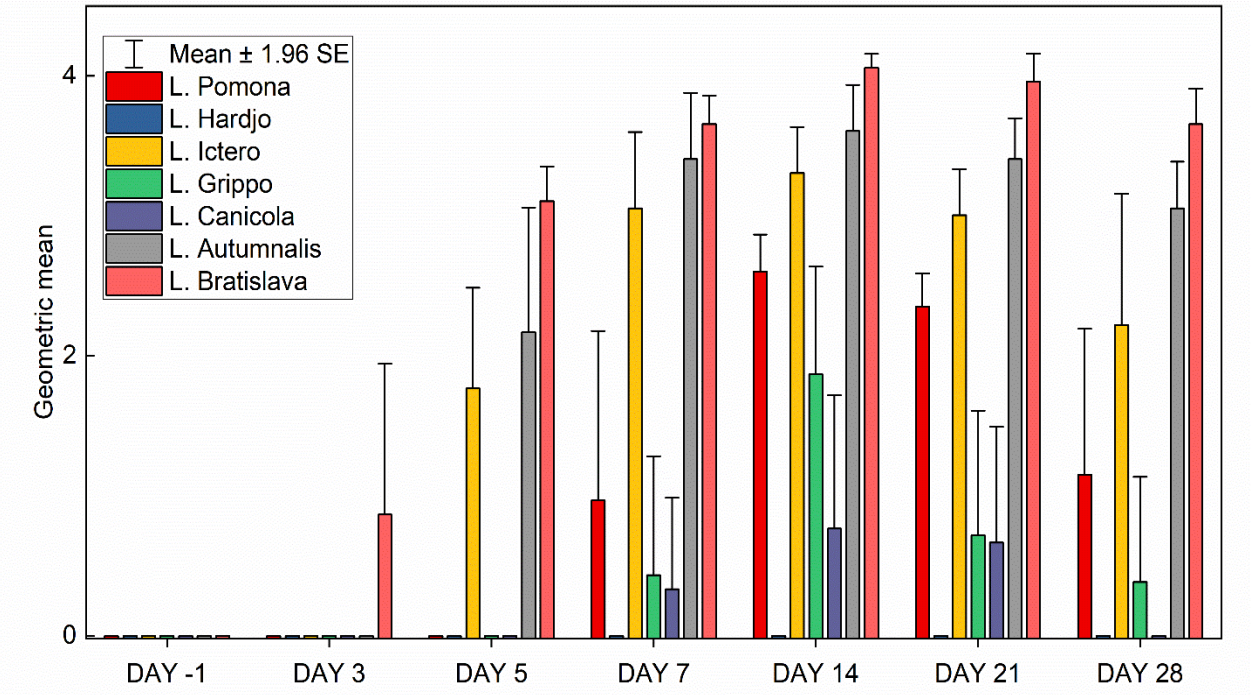

Figure 2.7: Geometric mean of the Microscopic Agglutination Test (MAT). Geometric mean of the agglutination antibody titers against 7 serovars of *Leptospira spp.* on each sampling date. Data presented is only from the challenged group and bars represent the geometric mean (Ln scale).

Horses from the control group remained negative for all seven tested serovars. The challenged group, on the other hand, based on the MAT test, presented cross-reacting antibodies as it can be seen on Figure 2.4 where MAT titer were also observed against other serovars than the one used in this challenge. The natural-log of MAT titers from each serovar was compared by ANOVA followed by Tukey test, the respective P-values are displayed on Table 2.1.

Table 2.1 Pairwise analysis of cross-reactivity among different serovars over time.

SEROVAR	DATE	*L.* Hardjo	*L.* Ictero	*L.* Grippo	*L.* Canicola	*L.* Autumnalis	*L.* Bratislava
		Tukey test (p-value)					
L. Pomona	-1	1.00	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00	0.07
	5	1.00	0.00	1.00	1.00	0.00	0.00
	7	0.44	0.00	0.92	0.84	0.00	0.00
	14	0.00	0.48	0.43	0.00	0.12	0.01
	21	0.00	0.56	0.00	0.00	0.08	0.00
	28	0.17	0.24	0.61	0.17	0.00	0.00
L. Hardjo	-1	1.00	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00	0.07
	5	0.00	1.00	1.00	0.00	0.00	0.00
	7	0.00	0.97	0.99	0.00	0.00	0.00
	14	0.00	0.00	0.37	0.00	0.00	0.00
	21	0.00	0.45	0.53	0.00	0.00	0.00
	28	0.00	0.98	1.00	0.00	0.00	0.00
L. Ictero	-1	1.00	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00	0.07
	5	0.00	0.00	1.00	0.00	1.00	0.02
	7	0.00	0.00	0.99	0.00	0.99	0.87
	14	0.01	0.00	0.98	0.00	0.98	0.40
	21	0.00	0.00	0.92	0.00	0.92	0.15
	28	0.00	0.00	0.52	0.00	0.52	0.04
L. Grippo	-1	1.00	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00	0.07
	5	1.00	0.00	1.00	0.00	0.00	0.00
	7	1.00	0.00	1.00	0.00	0.00	0.00
	14	0.06	0.00	1.00	0.00	0.00	0.00
	21	1.00	0.00	1.00	0.00	0.00	0.00
	28	0.98	0.00	1.00	0.00	0.00	0.00
L. Canicola	-1	1.00	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00	0.07
	5	0.00	0.00	1.00	1.00	0.00	0.00
	7	0.00	0.00	1.00	1.00	0.00	0.00
	14	0.00	0.00	1.00	1.00	0.00	0.00
	21	0.00	0.00	1.00	1.00	0.00	0.00
	28	0.00	0.00	1.00	1.00	0.00	0.00
L. Autumnalis	-1	1.00	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00	0.07
	5	0.00	0.00	1.00	0.00	1.00	0.02
	7	0.00	0.00	0.99	0.00	0.99	0.87
	14	0.00	0.00	0.98	0.00	0.98	0.40
	21	0.00	0.00	0.92	0.00	0.92	0.15
	28	0.00	0.00	0.52	0.00	0.52	0.04

Discussion

For many decades, *L. interrogans* serovar Bratislava was thought to be a host-adapted serovar and maintained by horses [29], and this postulate is based on studies that have reported identification of the serogroup Bratislava based on seroagglutination [17, 28, 45] and restriction endonuclease analysis (REA) [19, 31, 46] related to clinical cases. The *L. interrogans* serovar Bratislava has never been genetically confirmed from any horse around the world, where no whole genome sequencing (WGS) was performed in any of the reported cases, even when isolation and REA was done. This study was performed in an attempt to finally ascertain ability of this serovar to infect the horse, using an approximation of a natural infection, although the routes used might not represent those in natural infection.

Previous studies have shown that in hamsters and mice, the serovar, bacterial dosage and route of infection are important factors that influence a study model [47-54], although none of these evaluations were performed in horses. This is the first report of an animal challenge using *L. interrogans* serovar Bratislava in horses and thus most of the parameters used in this work are based on group experience and previous equine challenge outcomes [5]. Questions have been raised regarding the significance of this serovar as a cause of equine leptospirosis. In this study, the goal was to answer some of the questions about the pathogenicity of this serovar following horse infection, and to better understand the serological profile during infection, providing some guidance for better serologic diagnosis in the future.

Bryans [55], in 1955, and Yan [5], in 2010, when challenging horses with *L. interrogans* serovar Pomona and Pomona Kennewicki respectively, noticed high fever within 1-3 days post-challenge. In contrast, none of the challenged horses with *L. interrogans* serovar Bratislava had pyrexia in the first week following infection. As with most extracellular bacterial infections, some alterations in immune effector cells are expected. Pinna *et al.*[34], in 2010, when analyzing sera from 130 horses with a history of poor reproductive performance, found that horses that had the highest titer against for *L. interrogans* serovar Bratislava also had an increase in WBC, lymphocytes and neutrophils. In this study, we saw similar alterations regarding hematological status. WBC and lymphocyte counts were significantly higher in the challenged group. This recruitment of innate and adaptive immune effector cells can explain why the bacteria were cleared from the blood stream within less than 3 days. We were not able to see any significant alteration in the neutrophil counts.

Pinna *et al.* [34] also reported that when analyzing biochemical changes in the blood, the results showed that horses considered seropositive for *L. interrogans* serovar Bratislava exceeded the limits for creatinine, ALP, AST and total and direct bilirubin. In this study, we saw a significant difference (increased) between the challenged and the control group for total bilirubin and GLDH, and the concentration of iron in the blood of the challenged horses was significantly lower than the control group; however, the levels were within normal reference ranges. These changes were attributed to acute inflammation (iron), mild hepatitis (GLDH) and a possible decrease in appetite (bilirubin).

In this work, two methods for the detection of leptospires were used: Culture and PCR. Culture was not able to isolate any bacteria, and PCR was universally negative, failing to confirm colonization. The diagnosis of leptospirosis based on culture of *Leptospira* is known to be difficult, time consuming and has a high rate of false negatives [22, 56]. Because the challenged horses developed a humoral response against *Leptospira* spp. along with some stimulation (changes) on the primary and immune factors, this might suggest that the bacterium had the chance of colonizing the host although it was rapidly cleared from blood, confirming the challenge infection. Nonetheless, because the detections methods used were not able to detect the bacteria in the blood, this might suggest that the animals were able to clear the bacteria within less than 3 days (when the first sample after inoculation was taken), or that the level of microorganisms left alive in the hosts were either absent or low in blood and urine and could not be detected by any of the used methods.

The MAT test showed an increase in the levels of antibodies against the challenge strain within 3 days post-challenge (Figure 2.4) and the beginning of a decrease in titer after 21 days. This is an expected immune response due to a transient infection. Serum from infected horses normally present high levels of agglutination antibodies against this serovar, leading to the suggestion of a supposed host-adapted serovar. Approximately 45% of the horses tested in North America present antibodies against *L. interrogans* serovar Bratislava [18, 20, 57], and the same serovar has been shown to be among the serum-prevalent groups in many countries in Europe [7, 17, 27, 58, 59] and around the

world [16, 34, 60-63]. However, in almost all of these studies, cross-reactivity was reported, which makes it difficult to determine the infecting serovar, once more challenging the concept that MAT can identify the infection serovar in equine leptospirosis.

In the present study, cross-reactivity was moderate to significant between some of the seven serovars tested depending on the sampling date (Table 2.1). *L. interrogans* serovar Bratislava showed higher titers than most of other serovar used in the MAT from day 3 until day 28 ($P<0.05$). MAT Titer against other serovars than Bratislava can be seen as earlier as day 5 (Ictero and Autumnalis) as showed in Figure 2.4 and are significantly higher than the titers for other serovars used in the MAT (Table 2.1). The *L. interrogans* serovar Autumnalis also showed significant higher MAT titers than most of the other serovars starting on day 5 until day 28 ($P<0.05$). In clinical cases, this cross-reactivity can easily mislead the diagnosis of the offending serovar, especially when early infection titers are used as an indicator of the infecting serovar. In general, during cases of leptospirosis, the host serum reacts serologically against many serotypes and serogroups when tested by the gold standard method MAT [15, 58, 63-67]. Cross-reactivity might be the reason why many researchers thought that *L. interrogans* serovar Bratislava was a host-adapted strain in horses due to the observation of high titers against this serovar in addition of serotyping classification of isolated samples.

In order to fill in the knowledge gaps and better understand of the cross-reactivity among these strains, a pangenomic analysis was performed using GView server (https://server.gview.ca)[68] that includes the Basic Local Alignment Search Tool (BLAST) Atlas method. This method was not intended as part of this work, so for this reason it is placed in the discussion section. Six *L. interrogans* strains representing the six serovars used in the MAT test show that around 97% (alignment length cutoff = 100bp and percent identity cutoff = 75%) of the *L. interrogans* serovar Bratislava genome is covered (Figure 2.5 and Supplementary Material S1).

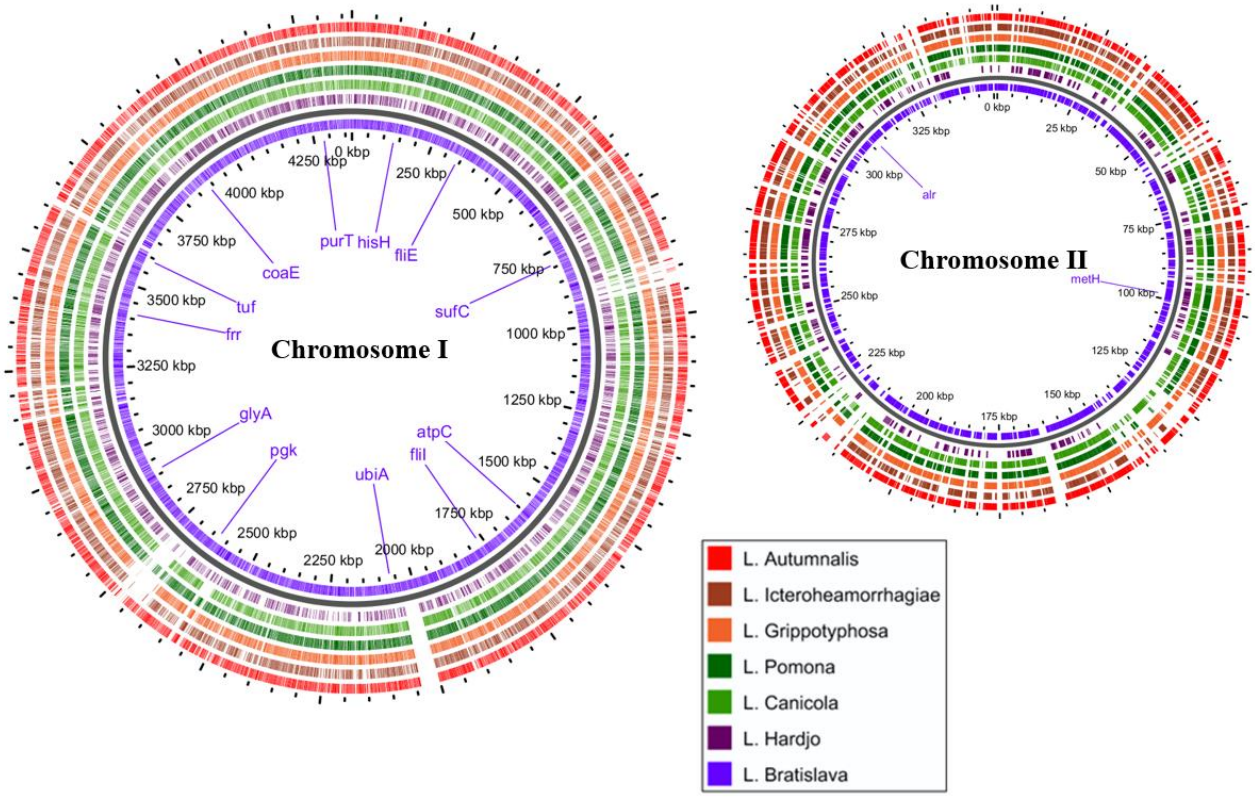

Figure 2.5: Pangenomic analysis of serovars used in the MAT test. Genomes were extracted from Patric - https://patricbrc.org). Genome: 1049795.3 - *L. interrogans* serovar Autumnalis str. LP101; Genome ID : 1049910.3 - *L. interrogans* serovar Icterohaemorrhagiae str. Verdun HP; GenomeID : 1049903.3 - *L. interrogans* serovar Grippotyphosa str. UI 08434; Genome ID : 1001587.6 - *L. interrogans* serovar Pomona str. Pomona; Genome ID : 211880.12 - *L. interrogans* serovar Canicola str. RUFN; Genome ID : 1279460.3 - *L. interrogans* serovar Hardjo str. Norma; and Genome ID : 338215.3 - *L. interrogans* serovar Bratislava strain PigK151 – used as the reference genome. The inner-most (blue) slots on the maps show the CDS regions on the reference genome. The outer slots show regions on the reference where there was a BLAST hit within one of the query files. Empty regions on the query slots indicate areas where there are no BLAST hits between the reference and the query files.

The data from the genomic analysis is available in the Supplementary Material section. As expected, around 31% (29 out of 97) of the “unique” features found on *L. interrogans* serovar Bratislava are enzymes with some of these being related to LPS biosynthesis and outer membrane formation. However, the other portion of genes were commonly found within the other strains, providing a likely explanation for the cross-reactivity seen in the MAT results.

Based on the genomic analysis and the animal challenge results, there is a suggestion that the *L. interrogans* serovar Bratislava is possibly not a host-adapted serovar for horses as it was first assumed, and most of the cross-reaction seen on serological profiles is due to widely shared genome features. These findings are important for the diagnosis of leptospirosis in horses, because misdiagnosis could lead to erroneous treatment that could harm the patient. These results might be extrapolated to other host species and are worth investigating. This more nuanced understanding combined with better genomic tools should be valuable in developing better diagnostic tests and novel vaccines that could provide protection against a broad range of serovars.

A better genomic screening might help to better select strains with lower cross-reactivity levels and higher host-specificity to build better MAT serovar (or serogroups) combinations. Furthermore, since the strain and serovar used in this work is suggested to not be pathogenic neither host-adapted for horses (did not cause clinical disease or colonization) but experimental infection resulted in

the production of agglutinating antibodies against other serovars (Figure 2.3 and 2.4), it would be valuable to conduct future studies investigating cross-protective attributes of this serovar in this host. This animal experimental challenge had two main limitations: 1) The results might have varied if another strain from the same serovar had been used and 2) The use of another route of infection and a higher bacterial dose might have achieved colonization. Further investigations on these aspects should be done prior to concluding that *L. interrogans* serovar Bratislava PigK151 is not pathogenic or host-adapted for horses.

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CHAPTER THREE

EVALUATION OF NEW LEPTOSPIRAL ANTIGENS FOR THE DIAGNOSIS OF EQUINE LEPTOSPIROSIS: AN APPROACH USING PAN-GENOMIC ANALYSIS, REVERSE VACCINOLOGY AND ANTIGENIC SELECTION

Introduction

Leptospira is estimated to be responsible for more than 1 million of human leptospirosis cases and 60,000 fatalities annually, mostly affecting rural or suburban impoverished populations in tropical and subtropical regions worldwide [1]. The current gold standard diagnostic test for leptospirosis, the microscopic agglutination test (MAT), detects anti-*Leptospira* immunoglobulin titers in human and animal serum at the serogroup (mostly reported by serovars) level [2, 3]. Generally IgM-targeted antibodies are produced within the first week of illness, allowing relatively early diagnosis and, thus, treatment [4]. The Centers for Disease Control and Prevention (CDC) defines a MAT titer of *Leptospira* agglutination titer of ≥800 being confirmative for *Leptospira* infection, although paired tests are likely to provide information on duration of infection [5]. MAT has a high diagnostic specificity but relatively low sensitivity for identifying agglutinating antibodies in the host serum [6].

Unfortunately, MAT is prone to a significant range of practical problems. These problems may include the need for well-trained technicians and rigid maintenance of *Leptospira* strains used in the test. Even then, this test is prone to generating false positives and ambiguous results regarding serovar identity

[6]. In addition the choice of serovars to be included in the antigen panel affects the sensitivity of the assay, the WHO listed and recommends a detailed variety of serovars to be included in the battery of antigens for optimal testing of unknown samples [7]. During the early (acute) stage of infection, cross-reactivity among serovars can be a problem when MAT is used to identify the serovar causing infection, as reported on the literature [8-12]. Despite these disadvantages, MAT has high specificity when used at the immune stage or convalescent phase of infection.

However, most of time it is impossible to know exactly the phase of infection in clinical leptospirosis cases and as a serological test, MAT is really testing exposure, and that along with clinical signs would help determine the disease (leptospirosis). A search for a better diagnostic test that utilizes new targets and improves diagnostic test characteristics is crucial. Researches have been using reverse vaccinology (RV) for over two decades in a variety of ways: identifying serogroup B meningococcal vaccine candidates [13], analysis of pneumococcal virulence factors and identifying vaccine targets for *Streptococcus pneumoniae* [14-16], *S. agalactiae* [17] and *L. interrogans* [18]. Zeng *at al*. [19] demonstrated that applying RV using a negative-screening coupled with a Pan-Genomic analysis of 17 worldwide pathogenic strains of *L. interrogans* contributed to the antigen prediction of potential vaccine candidates against leptospirosis and also provided further insights into the mechanisms of leptospiral pathogenicity. Zeng *et al*. also reported 861 “unique” proteins among the tested strains of *L. interrogans*. These proteins were present in only one of the analyzed strains,

which lead to the hypothesis that some of these proteins could be used in a diagnostic test that may perhaps would differentiate infection at the strain or serovar level, similar or better than the MAT test.

In this study, Pan-genomic analysis data from 861 unique proteins and 7 core proteins reported by Zeng *et al*. [19] was evaluated and an antigenic selection approach was used to identify new leptospiral targets. Based on bioinformatics analysis, 8 probable antigenic targets from this list were produced and serologically tested for their ability to improve the diagnosis of equine leptospirosis. A set of new leptospiral antigens are proposed for diagnostic of equine leptospirosis.

Methods

Selection of antigens

The 861 unique proteins can be found in the Supplementary Material online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00396/full#supplementary-material. Protein and DNA information were extracted from Patric – (https://patricbrc.org) using the locus tag provided by the reference work. The strains and serovars used in the reference study were: *L. interrogans* serovar Australia str. 2002000624; *L. interrogans* serovar Bataviae str. 2006006976; *L. interrogans* serovar Bataviae str. L1111; *L. interrogans* serovar Bataviae str. UI 08561; *L. interrogans* serovar Bulgarica str. Mallika; *L. interrogans* serovar Copenhageni str. Fiocruz L1-130; *L. interrogans* serovar Grippotyphosa str. UI 08368; *L. interrogans* serovar Grippotyphosa str. UI 08434; *L. interrogans* serovar Grippotyphosa str. UI 12764; *L. interrogans* serovar Lai str. 56601; *L. interrogans* serovar Manilae str. M001_Tn_Mutant_Parent; *L. interrogans* serovar Medanensis str. UT053; *L. interrogans* serovar Muenchen str. Brem 129; *L. interrogans* serovar Pomona str. Pomona; *L. interrogans* serovar Pyrogenes str. 2006006956; *L. interrogans* serovar Pyrogenes str. L0374; and *L. interrogans* serovar Pyrogenes str. SriLanka1. All the information from the 861 proteins, the selections and scores are available in the Supplementary Material S3.

Bioinformatics Tools Used in Reverse Vaccinology

The schematic bioinformatics for antigenic selection approach used is shown in Figure 3.1.

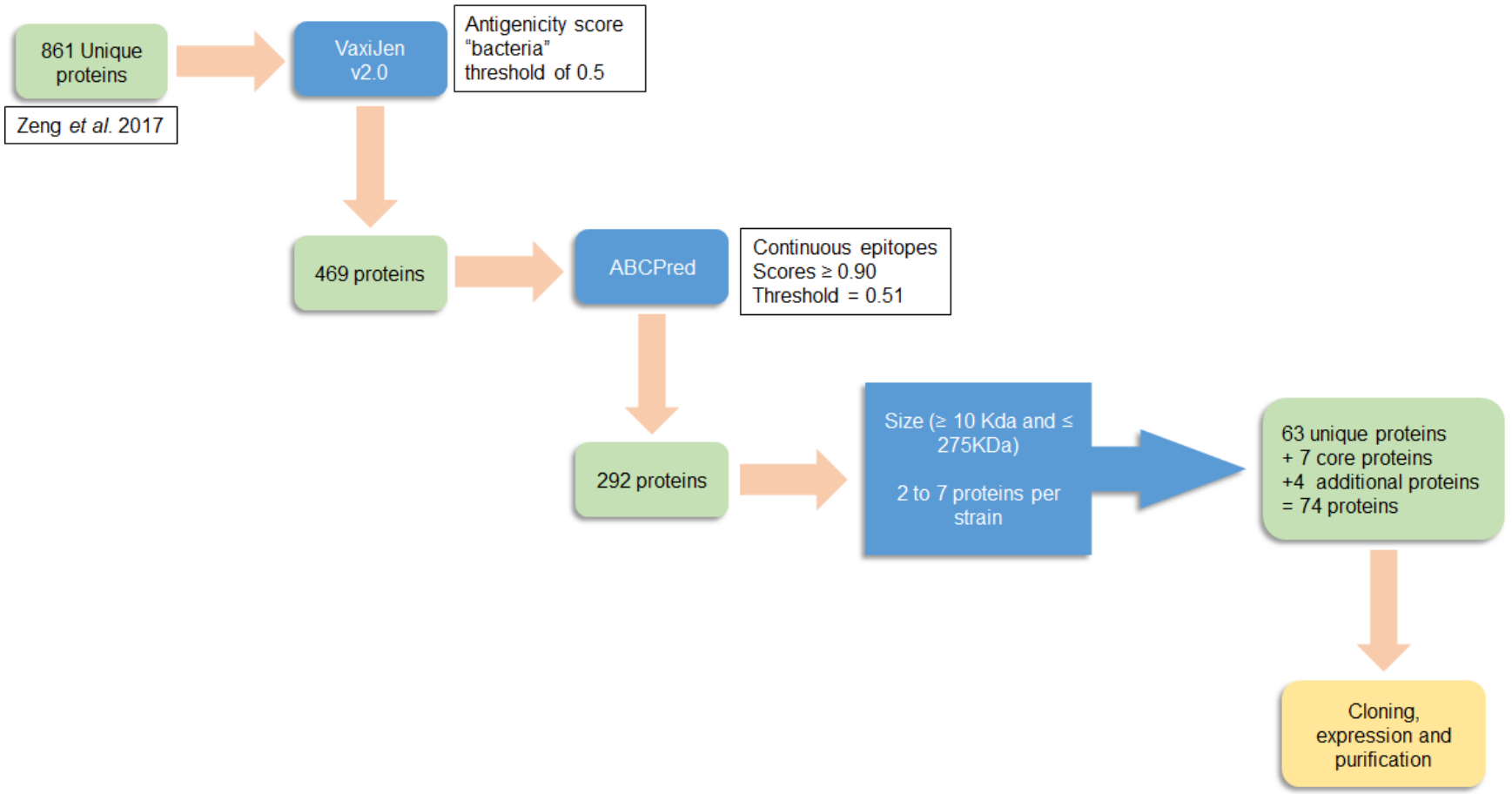

Figure 3.1: Schematic representation of the novel strategy of reverse vaccinology applied to unique proteins from 17 pathogenic *L. interrogans*.

The first step was prediction of the antigenicity score for each of the 861 proteins using VaxiJen server [20] with default parameter “bacteria” and a threshold of 0.5. Of these, 467 had a score ≥0.5 for bacterial antigen parameters, which were further explored for the presence B cell epitopes using ABCPred server [21]. Only 292 proteins had one or more putative B cell epitopes and were selected for further analysis. Next, to ease protein expression in a heterologous system, size limits of ≥10 and <275KDa were used. A total of 63 unique proteins were selected in a way that each strain, for all the 17 strains of *L. interrogans* mentioned in the reference work, between 2-7 proteins were selected for representing the respective strain. Seven additional proteins were added to this list which are shared among all 17 *L. interrogans* strains as reported by Zeng *at al*. [19]: LIC_10313; LIC_10760; LIC_10920; LIC_11067; LIC_11823; LIC_11846; LIC_11969; LIC_12938; LIC_13050; LIC_13417; LIC_20042. Some of these additional proteins were suggested to be important and virulence-related with some presenting high antigenicity scores.

In order to evaluate and compare the possible serum-reactivity of these new proteins, four previous reported proteins were included as core positive controls: $LigA_{4\text{-}8}$, LipL32, Loa22 and LipL21 [22, 23]. Protein subcellular localization was predicted by CELLO server [24] and proteins were classified into cytoplasmic proteins (CY), inner membrane proteins (IM), periplasmic proteins (PP), outer membrane proteins (OM), or extra-cellular proteins (EC).

Cloning, expression, and purification of the recombinant proteins

The *Leptospira* strains, primers and restriction sites used are available in the Supplementary Material S3. A Basic Local Alignment Search Tool (BLAST - https://blast.ncbi.nlm.nih.gov/) using nucleotide and protein sequences from each of the 70 selected genes was performed to identify compatible available *Leptospira* strains (in the lab) to be used as template. Due to restriction of strain availability, only 66 of 70 genes were targeted and gene amplification was carried out by PCR using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific). The strains used and the number of DNA targets from each respective strain were: *L. interrogans* serovar Bratislava strain PigK151 (48 targets /66 total); *L. interrogans* serovar Lai str. 56601 (10/66), *L. interrogans* serovar Canicola str. Fiocruz LV133 (2/66); *L. kirschneri* serovar Grippotyphosa str. RM52 (3/66) and *L. interrogans* serovar Bataviae str. L1111 (3/66). The enzyme restriction sites used for cloning into pET28a (Invitrogen) were: NcoI and XhoI (62/66), XbaI and XhoI (2/66), or NcoI and HindIII (2/66); allowing for a 6-HisTag at the C' terminal position of each target. Amplicons from PCR were checked by agarose gel electrophoresis and purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) (data not shown). Gel purification products and pET28a were digested with respective enzymes (ThermoFisher Scientific) for 2 hours at 37°C, followed by inactivation for 20 minutes at 80°C. Inserts and vector were ligated using T4 DNA Ligase (Invitrogen) at room temperature overnight. The obtained recombinant gene was transformed into *Escherichia coli* DH5α as the host strain. The DNA insert of each clone was verified by DNA

sequencing using T7 Promoter (forward primer: taatacgactcactatagg) and T7 terminator (reverse primer: caaaaaacccctcaagacccg) (data not shown).

The correct recombinant plasmids were then transformed into *E. coli* BL21(DE3) (Stratagene, Santa Clara, CA) for protein expression. The protein expression screening was done by dilution of 10 mL of overnight culture (clones into *E. coli* BL21(DE3)) into 100 mL of Terrific Broth (TB: 12 g tryptone, 24 g yeast extract, 0.017M of KH_2PO_4, 0.072M of K_2HPO_4, 12 mL glycerol, per liter) supplemented with 2 g/L of α-lactose (TB Lac^+). Protein expression was carried out for 24 and 48 hours and proteins were checked for positive expression by SDS-PAGE gel electrophoresis (data not shown). The expressed proteins were purified by chromatography using HisTrap HP columns (GE Healthcare Life Sciences) on an automated system AKTA Pure (GE Healthcare Life Sciences). The concentration of purified protein was then determined by $A280_{nm}$ on a NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific). Finally the proteins were screened on enzyme-linked immunosorbent assay (ELISA). The additional core positive controls ($LigA_{4-8}$, LipL32, Loa22 and LipL21) were produced as previously described [22, 23].

Sera

Horse sera were collected from January to July (2019) by the New York State Animal Health Diagnostic Center (AHDC), Cornell University, Ithaca, NY. These serum samples were either positive (96) or negative (32) in the MAT test against the following *Leptospira* serovars: *L*. *interrogans* serovar Pomona*, L. borgpetersenii* serovar Hardjo, *L*. *interrogans* serovar Icterohaemorrhagiae, *L*.

kirschneri serovar Grippotyphosa, *L*. *interrogans* serovar Canicola, *L*. *interrogans* serovar Autumnalis and *L. interrogans* serovar Bratislava.

Optimization of antigen concentration in ELISA assay

For each antigen, purified protein was diluted in coating buffer (0.05 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.6) to a final concentration of 20 µg/mL, placed into 96-well plates and a two-fold serial dilution was used un to achieve a final concentration of 0.1 µg/mL. Plates were incubated at 37˚C for 2 hours, followed by blocking with 3% bovine serum albumin (BSA) in PBS overnight. Plates were then washed 3 times with PBS-T 0.05% (PBS, 0.05% Tween 20). A positive serum (MAT = 12400) and a negative serum (MAT = 0) were used as serum controls. In order to establish optimum dilution, sera were diluted in PBS containing 1% BSA as follows: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400; then added to the wells for 1 hour at 37˚C. The plates were washed 3 times with PBS-T 0.05% and IgG reactivity was detected with Goat Anti-Horse IgG Polyclonal Antibody peroxidase-labeled (KPL, Inc. ML) and TMB 2-Component microwell peroxidase substrate (KPL, Inc. ML). The reaction was stopped after 10 minutes with 100 µL of 1M of H_3PO_4 and plates were read at OD_{450nm} on a microtiter plate reader (BioTek, VT).

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was performed as previously described [22, 23]. Briefly, purified proteins were diluted in coating buffer (0.05 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.6) at optimum concentration established by checkerboard titration. First, 100 µL of the diluted antigen were coated onto 96-well high-binding microtiter plates (Santa Cruz Biotechnology, Inc.) and incubated at 37˚C for 2 hours, followed by blocking with 200 µL of 3% BSA in PBS. After washing 3 times with PBS-T 0.05%, 100 µL of 1:800 diluted sera in PBS containing 1% BSA was added to the wells for 1 hour at 37˚C. The reactivity was detected as previously described.

Genetic sequence analysis and antigen-specie specificity

A BLAST using nucleotide and protein sequences was used to identify the presence of the new recombinant proteins in the serovars used in the MAT test. Additional pathogenic, intermediate and saprophytic species were added to this analysis in order to identify the presence or absence of the antigens in additional strains. The references used for BLAST were: *L. interrogans* serovar Autumnalis (taxid:174157); *L. interrogans* serovar Bratislava (taxid:338215); *L. interrogans* serovar Canicola (taxid:211880); *L. kirschneri* serovar Grippotyphosa (taxid:38345)*, L. borgpetersenii* serovar Hardjo (taxid:328971); *L. interrogans* serovar Icterohaemorrhagiae (taxid:90062); *L. interrogans* serovar Pomona (taxid:44276); *L. biflexa* (taxid:172); *L. wolbachii* (taxid:29511); *L. meyeri* (taxid:29508); *L. vanthielii* (taxid:293085); *L. terpstrae* (taxid:293075); *L. kirschneri* (taxid:29507); *L. santarosai* (taxid:28183); *L. weilii* (taxid:28184); *L. inadai* (taxid:29506); *L. broomii* (taxid:301541) and *L. licerasiae* (taxid:447106).

The cutoff parameter for BLAST results were: percent identity: 50-100%, query coverage: 25-100%, gaps allowed: 25% and e-value=1e-05.

Data and statistical analysis

Statistical analysis was performed using Origin(Pro), Version 2018 software (OriginLab Corporation, Northampton, MA, USA). Positive (for at least one serovar, MAT titer ≥100, n = 96) and negative MAT (for all serovars, (MAT negative = 0, n = 32) samples were separated into two groups and screened for each protein. ELISA data was normalized to ensure a consistent scale (0-1) for results from several experimental runs. A cutoff parameter for distinguish ELISA positive from negative samples was the mean of the negative samples plus 1.96 standard error of the negative group ($\bar{X}_{MAT\_negative} + 1.96 * SE_{MAT\_negative}$). This cutoff parameter allows for the inclusion of 95% CI of the observations around the negative mean and anything greater than the upper limit of the 95% CI ($\bar{X}$ +1.96*SE) of the negative sample group mean will be consider as belonging to another population, in this case belonging to the positive group. A two-tailed t-test was used to identify significant differences ($P<0.05$) between group means (MAT positive and negative) for each antigen and considering a not-equal variance between groups (Welch Correction). The relative sensitivity (Se) and specificity (Sp) with 95%CI, positive predictive value (PPV), negative predictive value (NPV) and accuracy of ELISA for the detection of anti-*Leptospira* antibodies in equine sera were calculated in comparison to the MAT as the reference method (gold standard). MAT results were considered as the true state of its respective sample (infected and non-infected). Overall accuracy was calculated based on

the number of true positive (MAT and ELISA positive) and true negatives (MAT and ELISA negative) detected by each antigen over the total sample size (n = 128). The ELISA sensitivity and specificity were also evaluated using the four most accurate antigens mixed together on a series and parallel mode.

Results

Expression of 8 new leptospiral antigens

Overall cloning results are available in the Supplementary Material S3. Only 8 of the new recombinant proteins were successfully expressed and purified in addition to the 4 core controls. Data from each protein are shown in Table 3.1. The new leptospiral antigens will be mentioned throughout this work by the locus tag, this is the first report on the evaluation of these antigens and none of them has been characterized previously.

Antigen optimization and sera dilution

Based on titration, a protein concentration of 2.5 µg/mL was found as optimal concentration for $LigA_{4-8}$, and 20 µg/mL for all the other antigens for performing the assay. A serum titer dilution of 1:800 was selected as the optimum dilution.

Evaluation of ELISA with new antigens in comparison with MAT

Tests characteristics for each antigen are presented in Table 3.2. Thirty-two negative and 96 positive serum samples were used in this experiment (128 total serum samples). All 8 novel recombinant proteins reacted with MAT-positive equine serum samples, as well as the 4 positive controls as shown in Figure 3.2.

The characteristics of each ELISA result set are presented in Table 3.2. Only one protein (WP_061243705) did not present a significant difference ($P = 0.08$) between MAT negative and positive sample groups. For multi-antigen evaluation, only 4 proteins were selected based on higher accuracy scores: LA_0711 (92%), LA_1567 (91%), LIC11823 (89%), LEP1GSC077_3193 (91%).

Table 3.1: Detailed information of the eight recombinant proteins from *L. interrogans*

LOCUS	NCBI Reference Sequence	VaxiJen Bact	SIZE (bp)	SIZE (~Kda)	Feature	STRAIN Cloned from	CELLO v.2.5 server
LA_0711	NP_710892	0.544	984	37	Hypothetical protein	*L.* interrogans serovar Lai str. 56601	EC
LA_1567	NP_711748	0.579	750	28	Putative lipoprotein	*L. interrogans* serovar Bratislava strain PigK151	EC
LIC11067*	WP_001278842.1	0.838	326	13	Cytoskeletal protein CcmA	*L. interrogans* serovar Bratislava strain PigK151	CY
LIC11823	WP_000622504.1	0.533	1437	54	Conserved hypothetical protein with FHA domain	*L. interrogans* serovar Bratislava strain PigK151	OM
WP_061243705	WP_061243705.1	0.526	900	34	PAS domain S-box protein	*L. interrogans* serovar Bratislava strain PigK151	CY
LEP1GSC077_3193	EKO07525.1	0.675	444	17	Putative lipoprotein	*L. interrogans* serovar Bratislava strain PigK151	EC
LEP1GSC019_0154	EKR16348.1	0.650	279	11	Putative lipoprotein	*L. interrogans* serovar Bratislava strain PigK151	PP
LEP1GSC088_1171	EMN47584.1	0.513	651	25	Putative lipoprotein	*L. interrogans* serovar Bratislava strain PigK151	OM

*The protein CcmA (locus: LIC11067) is shared among all 17 strains of *L. interrogans*. Protein subcellular location: cytoplasmic proteins (CY), inner membrane proteins (IM), periplasmic proteins (PP), outer membrane proteins (OM), or extra-cellular proteins (EC).

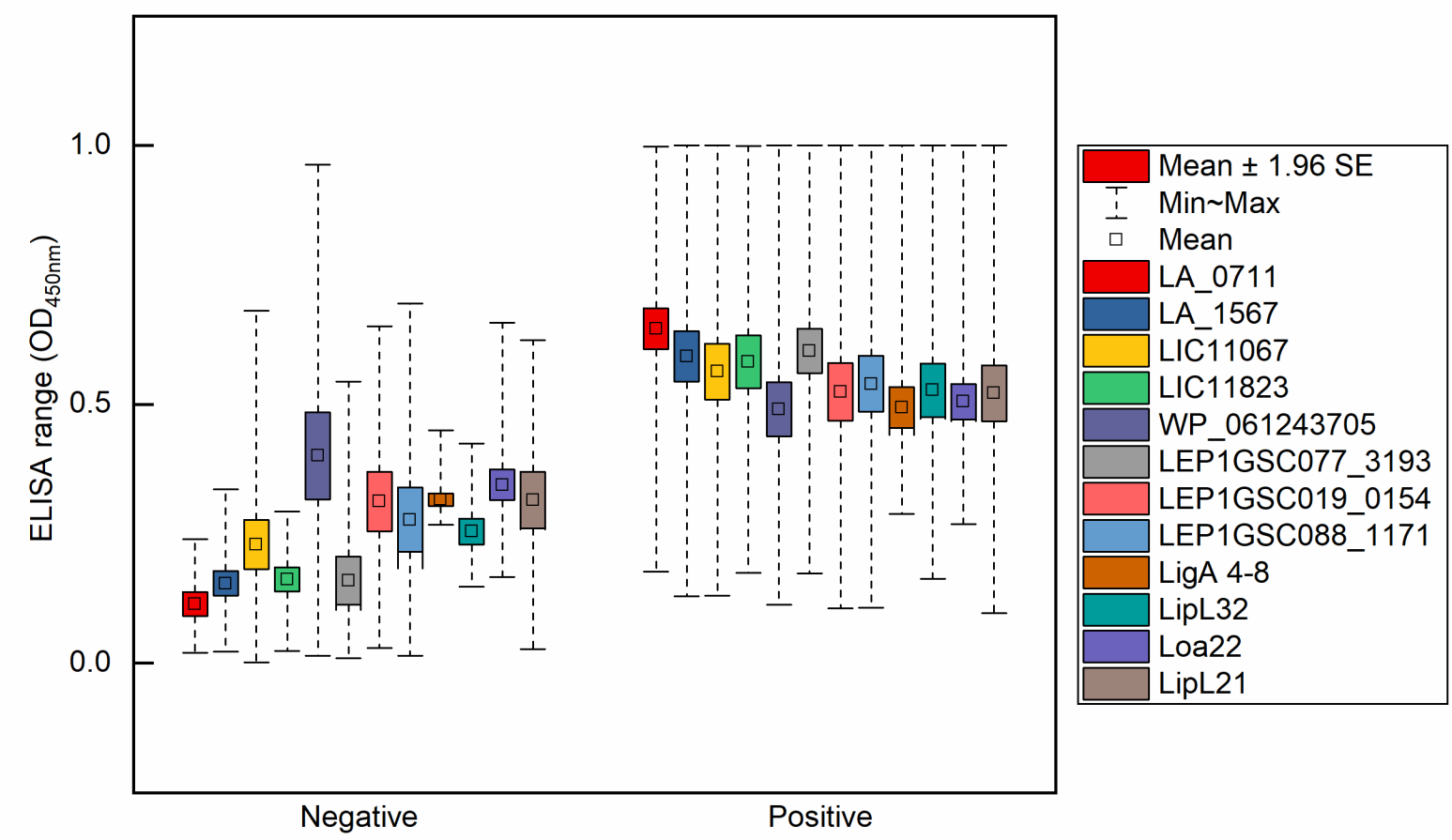

Figure 3.3: ELISA reactivity of 12 proteins according to MAT results. Data presented based on MAT positive samples and MAT negative samples.

Table 3.2: Antigen test characteristics on a serological screening compared to MAT.

	MAT		Test parameters compared to MAT					
LOCUS	**Mean Positive (95%CI)**	**Mean Negative (95%CI)**	**Se (95%CI)**	**Sp (95%CI)**	**ACCURACY**	**PPV**	**NPV**	**P-value* (pos x neg)**
LA_0711	0.646 (0.686-0.606)	0.114 (0.137-0.091)	100% (100%)	69% (85-53%)	92%	91%	100%	0.00
LA_1567	0.593 (0.642-0.544)	0.154 (0.178-0.130)	98% (100-95%)	72% (87-56%)	91%	91%	92%	0.00
LIC11067	0.563 (0.618-0.509)	0.229 (0.277-0.181)	79% (87-71%)	69% (85-53%)	77%	88%	52%	0.00
LIC11823	0.582 (0.634-0.531)	0.162 (0.185-0.139)	98% (100-95%)	63% (79-46%)	89%	89%	91%	0.00
WP_061243705	0.490 (0.542-0.438)	0.400 (0.485-0.316)	45% (55-35%)	72% (87-56%)	52%	83%	30%	0.08
LEP1GSC077_3193	0.603 (0.646-0.560)	0.160 (0.205-0.144)	97% (100-93%)	75% (90-60%)	91%	92%	89%	0.00
LEP1GSC019_0154	0.524 (0.580-0.468)	0.313 (0.370-0.255)	66% (75-56%)	65% (85-53%)	66%	86%	40%	0.00
LEP1GSC088_1171	0.540 (0.593-0.486)	0.277 (0.339-0.215)	72% (81-63%)	72% (87-56%)	72%	88%	46%	0.00
LigA4-8	0.494 (0.534-0.455)	0.316 (0.328-0.303)	82% (90-75%)	78% (92-64%)	81%	92%	60%	0.00
LipL32	0.527 (0.579-0.475)	0.254 (0.279-0.230)	79% (87-71%)	66% (82-49%)	76%	87%	51%	0.00
Loa22	0.505 (0.539-0.471)	0.344 (0.374-0.315)	75% (84-66%)	72% (87-56%)	74%	89%	49%	0.00
LipL21	0.521 (0.576-0.467)	0.315 (0.370-0.261)	56% (66-46%)	63% (79-46%)	58%	82%	32%	0.00

*P<0.05, MAT positive and negative groups compared by 2 tail t-test. Underlined accuracy scores were used in the multiple-antigen test.

Multiple-antigen combination

Parallel analysis: When at least one of two to four proteins tested positive when tested at the same time, the ELISA results were considered positive. **Series analysis:** When two to four proteins used sequentially and all tested positive, the ELISA result was considered positive. The results are available in Table 3.3.

Genetic analysis and accession of antigens serovar/species-specificity

BLAST revealed that 5 out of 8 of the cloned recombinant proteins were only present in pathogenic strains of *Leptospira*, and 2 out of 8 proteins were present on non-pathogenic species as shown in Table 3.4. Complete BLAST information is available in Supplementary Material S3.

Table 3.3: Sensitivity and specificity of ELISA when multiple antigens were evaluated.

Antigen combinations (locus)	SERIES		PARALLEL	
	Se (95%CI)	Sp (95%CI)	Se (95%CI)	Sp (95%CI)
LA_0711 + LA_1567	98% (100-96%)	81% (91-72%)	100% (100%)	59% (76-42%)
LA_0711 + LIC11823	98% (100-96%)	88% (96-79%)	100% (100%)	44% (61-27%)
LA_0711 + LEP1GSC077_3193	97% (99-94%)	84% (93-75%)	100% (100%)	59% (76-42%)
LA_1567 + LIC11823	96% (99-93%)	81% (91-72%)	100% (100%)	53% (70-36%)
LA_1567 + LEP1GSC077_3193	96% (99-93%)	84% (93-75%)	99% (100-97%)	63% (79-46%)
LIC11823 + LEP1GSC077_3193	95% (98-92%)	88% (96-79%)	100% (100%)	50% (67-33%)
LA_0711 + LA_1567 + LIC11823	97% (99-95%)	88% (94-81%)	100% (100%)	44% (61-27%)
LA_0711 + LIC11823 + LEP1GSC077_3193	95% (97-92%)	94% (99-89%)	100% (100%)	41% (58-24%)
LA_1567 + LIC11823 + LEP1GSC077_3193	94% (97-92%)	88% (94-81%)	100% (100%)	44% (61-27%)
LA_0711 + LA_1567 + LIC11823 + LEP1GSC077_3193	94% (97-91%)	94% (98-90%)	100% (100%)	41% (58-24%)

Table 3.4: BLAST results* from 8 new leptospiral antigens against MAT serovars, pathogenic, intermediate and saprophytic species.

	MAT TEST SEROVARS							PATHOGENIC			INTERMIDIATE			SAPROPHYTIC				
LOCUS	*L. interrogans* serovar Autumnalis (taxid:174157)	*L. interrogans* serovar Bratislava (taxid:338215)	*L. interrogans* serovar Canicola (taxid:211880)	*L. interrogans* serovar Grippotyphosa (taxid:280498)	*L. borgpetersenii* serovar Hardjo (taxid:328971)	*L. interrogans* serovar Icterohaemorrhagiae (taxid:90062)	*L. interrogans* serovar Pomona (taxid:44276)	*L. kirschneri* (taxid:29507)	*L. santarosai* (taxid:28183)	*L. weilii* (taxid:28184)	*L. inadai* (taxid:29506)	*L. broomii* (taxid:301541)	*L. licerasiae* (taxid:447106)	*L. biflexa* (taxid:172)	*L. wolbachii* (taxid:29511)	*L. meyeri* (taxid:29508)	*L. vanthielii* (taxid:293085)	*L. terpstrae* (taxid:293075)
LA_0711																		
LA_1567																		
LIC11067																		
LIC11823																		
WP_061243705																		
LEP1GSC077_3193																		
LEP1GSC019_0154																		
LEP1GSC088_1171																		

*A red square represents a hit (or multiple hits) found on the respective strain or species analyzed. A green square means no hit observed.

Discussion

Leptospirosis diagnosis is largely dependent on available and specific laboratory tests. Over the years, the use of different methods have been reported for leptospirosis diagnosis, including: MAT [3, 8, 25], bacterial culture [26], PCR [27], real time PCR [28], and histopathological examinations [29]. MAT is currently the “gold standard” test used for detecting leptospiral antibodies in which serum from suspected hosts reacts with live antigen suspensions of different *L.* serovars. MAT test can serve as basis to detect seroprevalence rate [30-32], to provide information on exposure rates and suspected infecting serogroups in the geographic region being studied [25, 26, 33, 34], but it does not present any critical information about the carrier or shedding status of the host. Although MAT usually presents high specificity, it is a labor-intensive method and not highly sensitive, having highly observed cross reactivity between different serovars [6]. The test is also expensive and laboratory workers can become infected as well because live organisms are used in the assay. Consequently, extensive efforts have being made for the development of a more sensitive and specific diagnostic tests for leptospirosis that would require less labor and resources.

Another serological alternative test that has been shown to be very promising for leptospirosis diagnosis is an enzyme-linked immunosorbent assay (ELISA) carrying leptospiral antigens. Many ELISAs using recombinant proteins have been shown to fit the diagnosis criteria for an accurate and valuable test, like *Leptospira* immunoglobulin-like proteins (LigA and LigB) [23, 35-39], and other

proteins: LipL32 [23, 40-42], Loa22 [23, 42], LipL21 [23, 42, 43], OmpL1 [43], LipL41 [42]. The ELISA has advantages of simplicity, short-test performance and sensitized microwell plates that may be kept for months before use, the ability of running multiple samples and/or multiple antigens in only one plate [44]. Although many on these proposed tests reported a good performance, these proteins are known to be present in most pathogenic and intermediate species, and some even in non-pathogenic species [45]. Creating a test with a more accurate ability of detecting infecting serovar would certainly strengthen the chances for a change on the gold standard test, improving the information gained out of the diagnostic test.

New proteins are been discovered and tested constantly in order to improve leptospirosis diagnosis and mostly helping to formulate protective vaccines [46-48]. Exposed proteins, membrane proteins, lipoproteins and extra-cellular proteins are potential targets for the host immune system, and therefore are likely to be antibody targets. One strategy for identifying new possible leptospiral antigens is the application of Reverse Vaccinology (RV). Although RV has been used for identifying leptospiral vaccine candidates [49-52], this is the first time the approach of selecting “unique” proteins from a pan-genomic analysis with different strains of *L. interrogans* to identify targets, and have these targets evaluated, has been reported. This work was done based on the hypothesis that the use RV would allow the identification of novel antigens, and most of them not being commonly found across pathogenic strains, these antigens could then be used in an ELISA to improve the sensitivity and specificity of this test, and

after genomic comparison across species, have its ability of identification of infecting serovar asserted.

In this study, 292 unique proteins were classified as potential leptospiral antigen with antigenicity scores ≥0.5 and at least one B-cell epitope and its amino acid sequence (Supplementary Material S3). To a first screen from this list, 64 proteins were selected for cloning and protein expression. As biomolecular work can be laborious and difficult, it is expected that not all genes would have been amplified from the PCR reactions and furthermore not all proteins would be well expressed at first. A total of 43 genes were successfully cloned (Supplementary Material S3). Based on leptospiral strain availability and successful protein expression, eight novel leptospiral targets from 43 clones were well expressed (data not shown). The 4 previous reported core antigens ($LigA_{4-8}$, LipL32, LipL21, and Loa22) were successfully expressed and all proteins were then serologically screened. The serological evaluation of these proteins with equine serum samples collected from the Animal Health Diagnostic Center (AHDC) at Cornell University showed reactivity for most of all proteins, suggesting that the used approach for detecting novel leptospiral antigens is suitable. The AHDC MAT routinely uses seven serovars that occur commonly in New York State for the diagnosis of equine leptospirosis. The MAT targets both IgM and IgG, but is skewed toward IgG [12]; therefore, the coated antigens were used to establish an ELISA for improved detection of specific IgG present in horse sera.

The cutoff points used for the MAT test vary in literature and may not always have been chosen deliberately to accommodate optimal test conditions. Overall, MAT is known to present low sensitivity (40-60%) but a considerably high specificity (95-100%) [6, 12, 33]. A good diagnostic test for leptospirosis should score high for both aspects. As all of the proteins presented in this work were evaluated based on MAT reports, it is impossible to confirm the exact sensitivity and specificity, although these results can be considered to be a good approximation. The previously four core control proteins reacted as expected [22, 23] and were used in the ELISA analysis to evaluate and ensure the reactivity of the new recombinant proteins and endorse proper testing. When the overall MAT results were considered, meaning that all samples with titer ≥100 were considered positive (n = 96), the new leptospiral antigens showed significant reactivity differences between MAT positive and negative sample groups (Fig. 3.2). Only one protein (locus: WP_061243705) could not significantly differentiate between MAT positive and negative sera (P = 0.08) (Table 3.2). This protein is predicted to be a cytoplasmic protein, which might be true due the fact that it did not react well on ELISA even considering that it has a reasonable antigenicity score. It is worth mentioning that these results were based on MAT results, and therefore cytoplasmic proteins would not be among the available leptospiral antigens from *in vitro* cultivation used for MAT.

Interestingly, we found that 23/32 MAT negative serum samples were positive by ELISA against one or more of our 12 antigens. Of interest, one negative sample (1/32) had positive serological results for 11/12 tested antigens, two

negative samples tested positive for 9/12 tested antigens, four negative samples tested positive for 8/12 tested antigens, two negative samples tested positive for 7/12 antigens, one negative had positive serological results for 5/12 antigens, five negative samples had positive serological results for 4/12 antigens, two had positive results for 3/12 antigens, four MAT negative samples reacted against 2/12 antigen and two negative samples reacted against 1/12 antigens. Only nine samples (out of 32 MAT negative samples) tested negative for ELISA for all antigens tested. This is expected, because some of these antigens may be not well expressed well *in vitro* and therefore MAT would not be able to detect antibodies in these serum samples [40, 53] and it might suggest that some of these proteins can be upregulated during infection, or this reactivity could simply be due to unspecific antibody-antigen reactions. In this case, a further follow-up of these individuals via culturing or another method would be recommended on those samples/patients.

The development of diagnostic tests using recombinant proteins as ELISA antigens for leptospirosis in humans and other mammals was reported previously [23, 35-37, 54]. Most of the reported serological tests used proteins that are commonly found in pathogenic strains of *Leptospira,* with sensitivities ranging from 40-90% and specificity ranging from 75-100%. The results showed in this work suggest that these new leptospiral antigens can be used as sensitive and specific serodiagnostic markers (Fig. 3.2, Table 3.2 and 3.3). When compared to overall MAT results, LA_0711 had the highest sensitivity (100%; 95%CI: 100%), correctly detecting leptospiral antigens on a 96/96 MAT positive

samples. On the other hand, LEP1GSC077_3193 had the highest specificity (75%; 95%CI: 90% to 60%), correctly detecting 24/32 MAT negative samples. The WP_061243705 had the lowest sensitivity (45%; 95%CI: 55% to 35%), while LIC11823 presented the lowest specificity (63%; 95%CI: 79% to 46%). A test with high sensitivity and specificity could have limited usefulness in general use depending on its predictive values, which can vary with the disease prevalence in the target population and other factors, such as the accuracy of the gold standard test being used in our comparison. Using MAT data to estimate prevalence (MAT positive considered as real exposed/infected sample), the reposted ELISAs presented high positive predictive values, ranging from 83 to 92%, which represents the probability of a patient being truly exposed given its positive result on ELISA. The negative predictive values ranged from 30 to 100%, showing a varied accuracy of the negative results. The best predictive values were obtained from the antigen LA_0711, with PPV of 91% and NPV of 100%, once more suggesting that this antigen is a strong candidate for developing an improved leptospirosis diagnostic test.

It is important to point out that one of the most influential factors on the calculation of the predictive values is disease prevalence. Our study was limited to patients that had their serum submitted to the AHDC, which does not represent the entire equine population or the real prevalence of the disease. Moreover, test accuracy, not only for ELISA but for MAT, has influence on the predictive values. In this study, MAT was considered being “perfect” and this consideration was used to ascertain the antigens test characteristics (Se, SP,

Accuracy, PPV and NPV), which may not hold true and therefore these factors were limitations of this part on the present study and further studies of these antigens should be done.

A multi-antigen approach using only the four-most accurate proteins presented outstanding results as shown in Table 3.3. When LA_0711 and LIC11823 were tested in series (both proteins reacting for a test result to be considered positive), these proteins showed great results, presenting a sensitivity of 98% and specificity of 88%. Furthermore, if the information from LA_0711, LIC11823 and LEP1GSC077_3193 was used in series, these proteins showed the best results with sensitivity of 95% and specificity of 94%. When a parallel testing approach is used, LA_1567 and LEP1GSC077_3193 showed the best results with sensitivity of 99% and specificity of 63%. These results show that a multi-antigen ELISA using these novel antigens is also suitable for use in an improved leptospirosis diagnostic test. These multiple combination can increase the test abilities on detecting true positives and true negatives samples depending on the setup, as it is shown in Table 3.3. To establish the real accuracy of these targets further tests should be considered, for example pairing them with bacterial isolation and or PCR methods.

Finally, to ascertain the ability of these targets to detect the infecting serovar a genomic analysis was performed (Table 3.4). BLAST results showed that 5/8 proteins (LA_0711, LA_1567, LIC11823, LEP1GSC077_3193 and LEP1GSC019_0154) were present only in the pathogenic strains, suggesting

that these proteins might be involved in pathogenicity. Three out of eight (LIC11067, WP_061243705 and LEP1GSC088_1171) were present in pathogenic and intermediate strains, but LIC11067 and WP_061243705 were also present in non-pathogenic strains. Interestingly, the protein with locus LA_0711 BLAST results showed only 55% of sequence coverage, 26.6% identity and e-value:2e-08, with only one epitope well shared among strains of *L. interrogans:* "PGPNHY" (Supplementary Material S3). This suggest that this epitope could be a suitable *L. interrogans* vaccine target, but it should have its real antigenicity and cross-protection evaluated. The protein with locus LEP1GSC077_3193 (accession number: EKO07525.1) matches part of the LenD protein, more precisely, 100% homology on the first 147 amino acids of its complete sequence (437 amino acids) from *L. interrogans* serovar Copenhageni (accession number: ABU88671). Based on the deposit sequence information, there is a chance that the current 5' and/or the 3' may need to extend or the current gene model may need to be merged with a neighboring gene model, which would lead to the possible LenD sequence match. Despite this possible sequence flaw, leptospiral endostatin-like proteins (Len proteins) from *Leptospira* have been shown to have characteristics that facilitate invasion and colonization of host tissues, and protect against host immune responses during mammalian infection [45, 55]. It is impossible to say whether these first 147 amino acids are the immunodominant region or not, but as they are recognized by antibodies (Fig. 3.2 and Table 3.2) it is worthy further investigation to evaluate its antigenicity.

The newly reported antigens were not found to be completely serovar/species specific, due the fact that some of these antigens were found in more than one serovar or species analyzed, although some of them could be used to help on the identification of infecting serovar (Table 3.3). For example, LA_0711 is only present in three of the seven serovars used in this MAT sera library, therefore it could be used to narrow down the cross-reactivity normally seen on MAT, once this antigen was not found in serovars Autumnalis, Hardjo, Icterohaemorrhagiae and Pomona. Another example, LEP1GSC019_0154 was only found in four of the seven serovars, as LA_0711, this protein was not found in the serovar Autumnalis and Hardjo, but it was also not found present in serovar Grippotyphosa. The fact that some of these proteins were not found in some serovars used in the present MAT, but were found reactive, lights the possibility for the development of a serological test that would allow serovar identification. The present analysis had some limitation, as for the strains used to represent the serovars and the limited number of different serovars and species included. These proteins might be present in different species or serovars that perhaps would be seroprevalent in another region, and further genetic screening should be done prior to ascertain species and serovar specificity in those cases.

In conclusion, the new leptospiral antigens described in this research could increase the sensitivity and specificity of ELISA for detection *Leptospira* exposure and for the detection of leptospirosis in horses along with support from extra clinical signs. These multi-antigen combinations may be able to replace or at least supplement the current MAT for the diagnosis of equine leptospirosis in

the near future, after further validation with more defined equine serum samples. One future goal is to test these antigens against specific serum from vaccinated and non-vaccinated horses and establish the ability of these antigens on differentiate between vaccination and natural leptospiral infection in the horse. In addition, it might be valid to test these antigens against different host sera and an extensive range of serovars, and further search for a “universal” leptospirosis test that could be used to confirm infection and infecting serovars regardless of the disease phase.

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CHAPTER FOUR

CONCLUSIONS

Leptospirosis is a serious worldwide neglected zoonotic disease. *Leptospira* spp. infects humans and animals, causing severe health problems as well as negatively impacting the economy. This work provides important information and new insights for the leptospirosis field, especially regarding to the equine leptospirosis and the discovery of new leptospiral antigens that might be directly involved with pathogenicity. For many years, horses were believed to be an accidental maintenance host for *L. interrogans* serovar Bratislava, and even in some rare cases, the bacteria would be linked to abortions and clinical manifestations.

For the first time, an experimental challenge with *L. interrogans* serovar Bratislava strain PigK151 in horses is reported. No clinical manifestation of the disease was noted; neither *Leptospira* recovery from blood, urine or tissues. Humoral responses were detected as early as 3 days post-challenge. Moreover, humoral response was also raised against many serovars, showing the evidence of cross-reactivity and that the gold standard diagnostic test should be revised and most possibly changed. A pangenomic analysis helped to answer the question about the cross-reactivity among serovars used in the MAT test at the New York Animal Health Diagnostic Center, where 97% of the *L. interrogans* serovar Bratislava strain PigK151 genome is covered by the six *Leptospira* serovars used in the MAT. Based on the findings presented in this work, there

is a suggestion that *L. interrogans* serovar Bratislava is not pathogenic for horses and it is not a host-adapted serovar, although these results might have differed if another strain from the same serovar had been used instead.

There is a need for a better diagnostic test for leptospirosis, for humans and for animals. The reliance on the gold standard test for leptospirosis, the MAT, can misrepresent the reality of a clinical case. The application of reverse vaccinology (RV) on the selection of new leptospiral antigens from *L. interrogans* has led to the discovery of new targets for leptospirosis diagnosis. The evaluation of these potential antigens for the diagnosis of equine leptospirosis has shown promising results.

The ELISA sensitivity of LA_0711 antigen displayed the highest value (100%), correctly detecting leptospiral antigens on a 96/96 MAT positive samples. LEP1GSC077_3193 presented the highest specificity value (75%), correctly detecting 24/32 MAT negative samples. Based on test accuracy scores of LA_0711 (92%), LA_1567 (91%), LIC11823 (89%), LEP1GSC077_3193 (91%), these antigens were used to build a multi-antigen combination test in series and parallel, allowing the reckoning of more than one antigen results prior to final diagnosis. The best combined results were from LA_0711 and LIC11823 tested in a series (both proteins reacting for a test result be considered positive) presenting a sensitivity of 98% and specificity of 88%, and from parallel testing using LA_1567 and LEP1GSC077_3193 with sensitivity of 99% and specificity of 63%.

This work shows the development of indirect ELISA tests using single and multi-antigen testing employing new leptospiral antigens as diagnostic antigens for equine leptospirosis, which could serve as basis for future works on different hosts and strains. The use of these 7 new antigens in the ELISA was found to be sensitive and specific, and the results coincided with the standard MAT results. These multi-antigen combinations may be able to replace or at least supplement the current MAT for the diagnosis of equine leptospirosis in the near future, after further validation with more defined equine serum samples. These antigens can now be tested against specific serum from vaccinated and non-vaccinated horses have their ability on differentiate between vaccination and natural leptospiral infection in horse established.

Some of the newly reported leptospiral antigens, locus: LA_0711, LA_1567, LIC11823, LEP1GSC077_3193 and LEP1GSC019_0154, were found only in the pathogenic species, which might suggest that these antigens could be involved in virulence and pathogenicity. None of the reported antigens were found to be completely serovar/species specific, but some might be able to help identifying infecting serovar, once some antigens were not found present in all analyzed serovars.

In addition, it might be valid to test these antigens against different host sera and further search for a “universal” leptospirosis test that could be used to identify infection/exposure and infecting serovars regardless of the clinical phase or host.