Universidade Estadual de Londrina

ROBERTA TORRES CHIDEROLI

DESENVOLVIMENTO DE ESTRATÉGIAS DE CULTURA PARA ISOLAMENTO DE LEPTOSPIRAS FASTIDIOSAS E USO DE BIOINFORMÁTICA PARA DESCOBERTA DE NOVOS ALVOS VACINAIS E DROGÁVEIS PARA CONTROLE DA LEPTOSPIROSE ANIMAL

Londrina
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Tese apresentada ao Programa de Pós-graduação em Ciência Animal da Universidade Estadual de Londrina - UEL, como requisito para a obtenção do título de Doutora.

Orientador: Prof. Dr. Ulisses de Pádua Pereira

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Às leptospiras...

Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no oceano. Mas o mar seria menor se lhe faltasse uma gota.

Madre Tereza de Calcutá

CHIDEROLI, Roberta Torres. **Desenvolvimento de estratégias de cultura para isolamento de leptospiras fastidiosas e uso de bioinformática para descoberta de novos alvos vacinais e drogáveis para controle da leptospirose animal**. 2020. 107f. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2020.

RESUMO

A leptospirose, causada por espiroquetas do gênero *Leptospira*, é uma zoonose globalmente disseminada, negligenciada e emergente. O isolamento de leptospiras é o primeiro passo para sua caracterização molecular e genotipagem, no entanto, para os sorovares mais fastidiosos, a cultura e isolamento são controversos e laboriosos. Com relação ao controle e tratamento da leptospirose, muitos estudos são realizados, mas ainda não existe uma vacina eficaz contra todas as espécies patogênicas ou variedade de antimicrobianos com atuação sobre o quadro de leptospirúria. Assim o presente estudo visou primeiramente desenvolver estratégias de cultura e isolamento para as leptospiras fastidiosas e utilizar métodos rápidos de diagnóstico molecular para caracterização e tipificação dos sorovares. Posteriormente, a partir do isolamento de novas estirpes, o sequenciamento genômico foi realizado com a finalidade de obtenção de dados de genômica comparativa com outras estirpes disponíveis no GenBank para uma busca *in silico* de alvos drogáveis e vacinais. No primeiro estudo, três formulações de meios de cultura líquidos básicos foram produzidas para isolar e manter leptospiras. Em cada formulação (A, B e C) foram adicionados diferentes suplementos para ajudar no crescimento de leptospiras fastidiosas: piruvato de sódio, enzima superóxido dismutase e soro fetal bovino. Durante o período aproximado de 55 dias, adotou-se estratégias de troca entre as três formulações de acordo com a observação em microscopia de campo escuro e ao final desse período houve sucesso no isolamento de três estirpes do sorovar Hardjo (dois genotipos, Hardjobovis e Hardjoprajitno) com adaptação total ao meio de cultura. Posteriormente, estas culturas foram avaliadas com uso da microscopia eletrônica e notou-se diferenças na morfologia e viabilidade de acordo com a composição do meio. No segundo estudo, os genomas desses dois genotipos foram sequenciados, montados e depositados no GenBank. A partir desses dados e também de outros genomas de outras espécies de *Leptospira* foi possível realizar uma abordagem de com base na vacinologia reversa, genômica comparativa e de *docking* molecular com o uso de ferramentas de bioinformática. Para os alvos vacinais, os resultados com base nas características da probabilidade de adesão, TMHMM e densidade de epítopos, sugerem que cinco alvos são bons candidatos e podem ser testados rapidamente em novas formulações de vacinas e posteriormente testados *in vivo*. Para a etapa de docking molecular, oito proteínas preditas como citoplasmáticas e identificadas como essenciais para a sobrevivência de leptospiras foram consideradas bons alvos para novos medicamentos. Destacando a proteína de divisão celular FtsZ, que foi o alvo identificado com as melhores características de ligação e, por meio de uma triagem virtual, o ZINC04259719 foi identificado como a melhor molécula para ligar a essa proteína, a fim de inibir sua função eliminando o patógeno. A vacinologia reversa e o *docking* molecular são poderosas ferramentas de bioinformáticas que possibilitam uma busca rápida e sem procedimentos laboriosos, por alvos proteicos específicos

que possam ser candidatos a uma nova vacina ou medicamento no controle da leptospirose.

Palavras-chave: Meio de Cultura. Caracterização Molecular. Genomas de *Leptospira*. Alvos Proteicos.

CHIDEROLI, Roberta Torres. **Development of culture strategies for isolation of fastidious leptospires and use of bioinformatics to discover new vaccine and drug targets for animal leptospirosis control**. 2020. 107 f. Thesis (Doctor´s Degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2020.

ABSTRACT

Leptospirosis, caused by spirochetes of the genus *Leptospira*, is a globally widespread, neglected and emerging zoonosis. The leptospires isolation is the first step towards its molecular characterization and genotyping, however, for the most fastidious serovars, the culture and isolation are controversial and laborious. Regarding the control and treatment of leptospirosis, many studies have been carried out, but there is not yet an efficient vaccine against all pathogenic species or more variety of antimicrobials acting on leptospiruria. Thus, the present study aimed primarily to develop culture and isolation strategies for fastidious leptospires and using rapid molecular diagnostic methods for characterization and typing of serovars. Subsequently, from the isolation of new strains, genomic sequencing was carried out in order to obtain comparative genomic data with other strains available at GenBank for an *in silico* search for drug and vaccine targets. In the first study, three formulations of basic liquid culture media were produced to isolate and maintain leptospires. In each formulation (A, B and C) different supplements were added to help the growth of fastidious leptospires: sodium pyruvate, superoxide dismutase enzyme and fetal bovine serum. During the approximate period of 55 days, exchange strategies were adopted between the three formulations according to the observation in dark field microscopy and at the end of this period, there was success in the isolation of three strains of serovar Hardjo (two genotypes, Hardjobovis and Hardjoprajitno) with total adaptation to the culture medium. Subsequently, these culture was evaluated through electron microscopy and differences in morphology and viability were noted according to the composition of the medium. In the second study, the genomes of these two genotypes were sequenced, assembled and deposited on GenBank. From these data and also from other genomes of *Leptospira* species, it was possible to carry out an approach based on reverse vaccinology, genomic comparative and molecular docking with the use of bioinformatics tools. For vaccine targets, results based on characteristics of probability of adherence, TMHMM and epitope density, suggest that five targets are good candidates and can be tested quickly in new vaccine formulations and subsequently tested *in vivo*. For the molecular docking step, eight proteins predicted as cytoplasmic and considered essential for the survival of leptospires were considered good targets for new drugs. Highlighting the cell division protein FtsZ, which was the target identified with the best binding characteristics and through a virtual screening the ZINC04259719 was identified as the best molecule for linked to this protein in order to inhibit its function and consequently eliminating the microorganism. Reverse vaccinology and molecular docking are powerful bioinformatics tools that enable a quick discovery, without laborious procedures, for specific protein targets that may be candidates for a new vaccine or therapeutic target to leptospirosis control.

Key-words: Culture Media. Molecular Characterization. *Leptospira* genome. Protein Targets

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LISTA DE ABREVIATURAS E SIGLAS

AA	Amino acid
BATS	Blast Automatic Targeting for Structures
BLAST	Basic Local Alignment Search Tool
BRIG	BLAST Ring Image Generator
BSA	Bovine serum albumin
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CIM	Concentração inibitória mínima
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CoA	Coenzyme A
DEG	Database of Essential Genes
ELISA	Enzyme Linked Immuno Sorbent Assay
EMJH	Ellinghausen-Mccullough-Johnson-Harris
GIPSy	Genomic Island Prediction Software
IEDB	Immune Epitope Database
ILS	International Leptospirosis Society
IS	Insertion Sequences
LGCM	Laboratory of Cellular and Molecular Genetics
LPS	Lipopolissacarídeos
LPSN	List of Prokaryotic names with Standing in Nomenclature
MHC	Major Histocompatibility Complex
MLST	Multiple Locus Sequence Typing
MLVA	Multilocus Variable-Number Tandem-Repeat Analysis
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequence
OMP	Proteína da Membrana Externa Putativa

PAI	Pathogenicity island
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PGAP	Prokaryotic Genome Annotation Pipeline
PSE	Putatively exposed to the surface
RFLP	Restriction Fragment Length Polymorphism
SAM	Soroaglutinação Microscópica
SEM	Scanning electron microscopy
TMHMM	Predict transmembrane helices in proteins
TTAR	Triagem tradicional de alto rendimento
TV	Triagem virtual
UEL	Universidade Estadual de Londrina
UFLA	Universidade Federal de Lavras
VFDB	Virulence Factor Database
VNTR	Variable Number Tandem Repeat
VR	Vacinologia Reversa

SUMÁRIO

1 INTRODUÇÃO

A leptospirose é uma zoonose de importância global causada por um grupo de bactérias pertencentes à ordem *Spirochetales*, família *Leptospiracea* e nos últimos anos tem sido considerada uma das principais doenças infecciosas emergentes (TRONCOSO; CASTRELO, 2016). Fouts et al. (2016) e Caimi et al. (2017) estabeleceram uma classificação de *Leptospira* por uma ampla comparação genômica interespecífica de todas as espécies infecciosas e não infecciosas conhecidas dessa bactéria. Hoje, o gênero é composto por espécies patogênicas e intermediárias, que causam leptospirose grave e leve, respectivamente, e espécies saprófitas de vida livre. Existem mais de 300 sorovares identificados em mais de 20 sorogrupos diferentes, divididos entre 23 espécies genômicas: 10 patogênicas, seis intermediárias e sete saprófitas, com último membro (*Leptospira venezuelensis*) adicionado em 2018 no grupo intermediário (PUCHE et al., 2018).

O agrupamento por métodos sorológicos é limitado, pois pode apenas distinguir os sorovares a nível de sorogrupo, mas não diferenciam os variados genotipos, como por exemplo, o sorovar Hardjo que é dividido geneticamente em *L. interrogans* sorovar Hardjo Hardjoprajitno e *L. borgpetersenii* sorovar Hardjo Hardjobovis (PICARDEAU, 2013). A classificação genômica, que separa sorovares em genotipos e espécies diferentes, é mais atual e baseada em estudos moleculares e de patogenicidade com uso de ferramentas como sequenciamento de próxima geração (NGS) e bioinformática (CERQUEIRA; PICARDEAU, 2009).

O isolamento de leptospiras lcutaé o primeiro passo para suac caracterização molecular e genotipagem; no entanto, para os sorovares mais exigentes, como por exemplo o Hardjo, a cultura e isolamento são controversos e laboriosos (PAILHORIÈS et al., 2015). Esses microrganismos são de crescimento lento e requerem um meio rico com pH neutro, o que dificulta o cultivo a partir de fontes naturais (STANECK et al., 1973; ADLER et al.,1986; GONZÁLEZ et al., 2006; ZACARIAS et al., 2008). Embora os meios de cultura comuns sejam adequados para recuperar as leptospiras menos exigentes, para o sorovar Hardjo as taxas de sucesso são mais baixas e estão relacionadas principalmente as suas exigências nutricionais peculiares (FLINT; LIARDET, 1980; ELLIS; THIERMANN, 1986; LEONARD et al., 1992).

Muitos mecanismos estão envolvidos na capacidade das leptospiras

de sobreviver nos rins, e acredita-se que a colonização dos túbulos renais proximais do hospedeiro promova a formação de agregados celulares e biofilme, e consequentemente, poucos medicamentos são eficazes na sua eliminação dos túbulos renais (RATET et al., 2014, KUMAR et al., 2016). Existem inúmeros benefícios que uma comunidade bacteriana pode obter formando biofilmes. Os biofilmes conferem resistência a muitos antimicrobianos, proteção contra protozoários e proteção contra as defesas imune do hospedeiro. Uma razão para a resistência ao estresse ambiental parece ser o aumento na porção de células persistentes dentro do biofilme (LOPÉZ et al., 2010).

Alguns estudos de susceptibilidade aos antimicrobianos concordam que não há uma característica de resistência nas leptospiras, apenas para sulfa trimetropim e polimixina (CHAKRABORTY et al., 2010; VANAPORN et al., 2013), entretanto, estudos mais recentes indicam um aumento na concentração inibitória mínima (CIM) para os antibióticos doxiciclina e tetraciclina (BENACER et al., 2017; LIEGEON et al., 2018). Contudo, é necessário ficar alerta à rápida disseminação dos mecanismos de resistência aos antimicrobianos, pois causa um impacto negativo no tratamento de doenças infecciosas (KRAEMER; RAMACHANDRAN; PERRON, 2019). Assim, é imprescindível mais pesquisas voltadas à descoberta *in silico* de novos alvos drogáveis ou produtos alternativos ao uso de antimicrobianos.

A vacinação de animais é o método de prevenção mais adotado em todo o mundo, entretanto, as vacinas comercias são constituídas de bacterinas com estímulo da imunidade limitado. Existem algumas controvérsias sobre o uso dessas vacinas com relação a sua proteção a longo prazo, capacidade de proteção contra a colonização renal, eliminação das bactérias do túbulo renal e quanto a diminuição das falhas reprodutivas em bovinos (ELLIS, 2015). Atualmente, as pesquisas buscam novos candidatos proteicos vacinais utilizando a abordagem de vacinologia reversa (VR). No entanto, para a leptospirose, embora existam algumas pesquisas para descobrir novos alvos proteicos vacinais (HARTWIG et al., 2011; HARTWIG et al., 2013), nenhum ainda foi implementado com sucesso (MURRAY et al., 2013; DELLAGOSTIN et al., 2017).

Atualmente, os estudos relacionados ao sequenciamento genômico de microrganismo, tem ganhado cada vez maior destaque na área de microbiologia médica e preventiva. Contudo, é essencial desenvolver estratégias de cultura para os sorovares mais fastidiosos, para assim realizar sua caracterização molecular. Com o

genoma disponível em bancos de dados públicos, é possível utilizar ferramentas de bioinformática para realizar estudos de genômica comparativa com a finalidade de descobrir proteínas relacionadas a virulência e patogenicidade, e desta forma, contribuir com avanços no tratamento e controle da leptospirose animal.

2 REVISÃO DE LITERATURA

2.1 TAXONOMIA E CLASSIFICAÇÃO

As leptospiras são classificadas dentro do filo *Spirochetes*, ordem *Spirochaetales*, pertencendo à família *Leptospiraceae*, composta pelos gêneros *Leptospira* spp., *Turneria* spp. e *Leptonema* spp. (ADLER; DE LA PENA MOCTEZUMA, 2010).

Desde sua descrição original em 1907, o gênero *Leptospira* é tradicionalmente dividido em dois grupos sorológicos: saprófitas - *Leptospira biflexa sensu lato* - e patógenos - *Leptospira interrogans sensu lato* - com base em sua patogenicidade e virulência (FAINE et al., 1999). Mais recentemente, a análise filogenética revelou que as leptospiras podem ser divididas em três grupos que se correlacionam com o nível de patogenicidade das espécies: saprófitas, intermediárias e patogênicas (KO et al., 2009).

Atualmente, segundo o comitê internacional de taxonomia das bactérias (LPSN – List of Prokaryotic names with Standing in Nomenclature), o gênero *Leptospira* compreende 28 diferentes espécies gênomicas: 12 pertencentes ao grupo patogênico (*L. interrogans, L. kirschneri, L. noguchii, L. borgpetersenii, L. alexanderi, L. weilii, L. santarosai, L. kmetyi, L. alstoni, L. mayottensis, L. stimsonii e L. yasudae*), sete ao grupo intermediário (*L. licerasiae, L. wolffii, L. fainei, L. broomii, L. inadai*, *L. venezuelensis e L. johnsonii*) nove ao grupo apatogênico (*L. idonii, L. meyeri, L. terpstrae, L. biflexa, L. vanthielii, L. yanagawae, L. wolbachii, L. ellinghausenii e L. ryugenii*) (PARTE, 2018).

Thibeaux et al. (2018) descreveram 12 novas espécies de leptospiras ambientais (saprófitas) e Vincent et al. (2019) realizaram um estudo onde foram sequenciadas 90 estirpes de *Leptospira* com uma comparação dos valores médios de identidade nucleotídica dos genomas e também dos genomas representativos de espécies já conhecidas. A partir destes dados este grupo revelou 30 novas espécies de *Leptospira* e os autores sugeriram a existência de dois clados (P - patogênicas e S- saprófitas) e 4 subclados (P1, P2, S1 e S2). Contudo essas novas espécies ainda não foram inclusas na lista de nomes de espécies leptospirais da Sociedade Internacional de Leptospirose, sendo a L. *venezuelensis* a última espécie inclusa (ILS-

Minutes of TSC meeting, November 2017).

2.2 ASPECTOS BIOLÓGICOS E MORFOLÓGICOS

As leptospiras são microrganismos aeróbios estritos e crescem muito bem em temperaturas de 28 a 30°C, possuem multiplicação e crescimento lentos, com tempo de geração em torno de oito a 12 horas, e são exigentes no que se refere a meios nutritivos. Uma cultura em meio líquido leva de cinco a sete dias para atingir crescimento satisfatório para ser utilizada como antígeno em testes de soroaglutinação microscópica (SAM). As leptospiras são pouco resistentes à luz solar direta, aos desinfetantes comuns e aos antissépticos; e no meio ambiente, os sorovares patogênicos requerem para sua sobrevivência solo com pH em torno de 7,2 a 7,4 e alta umidade. Todas as leptospiras são sensíveis a pH ácido de 6,8 ou menos, porém sobrevivem em condições alcalinas em pH entre 7,8 e 7,9 (FAINE et al., 1999).

As leptospiras são bactérias espiraladas, muito finas (0,1 µm de diâmetro) e com comprimento variando de 6 a 20 µm, apresenta as extremidades em forma de gancho e dois filamentos axiais (flagelos periplásmicos) que facilitam sua motilidade (CULLEN et al., 2004). A organização estrutural e a composição química dessas bactérias são semelhantes às de outras bactérias Gram-negativas: membrana externa que envolve toda a célula, os filamentos axiais denominados de flagelos periplasmáticos e os cilindros protoplasmáticos, que incluem a membrana celular e a capa de peptidioglicano da parede celular. Possui também um envelope externo que é composto por LPS (lipopolissacarídeos) e mucopeptídeos antigênicos (CHARON; GOLDSTEIN, 2002). A visualização de leptospiras em preparação a fresco só é possível por microscopia de campo escuro e de contraste de fase e também apresenta afinidade tintorial pelos corantes argênticos (GREENE et al., 2011).

2.2.1 Meios de Cultura

As leptospiras necessitam de condições especiais de cultivo em laboratório já que não utilizam açúcares ou aminoácidos como fonte energética, apesar de possuírem em seu genoma os genes codificantes para todas as enzimas

componentes da via glicolítica (NASCIMENTO et al., 2004a). A fonte de carbono e energia utilizada são obtidas dos ácidos graxos de cadeia longa com mais de 15 carbonos por meio da X-oxidação. Logo, o cultivo *in vitro* em meios de cultura artificiais devem disponibilizar facilmente CO_2 e ácidos graxos de cadeia longa (FAINE et al., 1999).

Existem vários meios de cultura artificiais para o isolamento e manutenção de leptospiras, podendo se apresentar tanto na forma líquida (a mais utilizada) quanto nas formas semi-sólida ou sólida (SOBOLEVA, 1983; ADLER et al., 1986; FAINE et al., 1999). O meio de cultura mais utilizado é o Ellinghausen-McCullough-Johnson-Harris (EMJH), que possui um composto chamado tween 40/80 (ácidos graxos complexados a sorbitol) como fonte de carbono e albumina sérica bovina (BSA) como agente detoxificante. Geralmente, utiliza-se uma suplementação do meio EMJH com 8-12% de soro de coelho levemente hemolisado como fonte de ferro, cianocobalamina, albumina, ácidos graxos e outros nutrientes (FAINE et al., 1999). As culturas crescem em temperatura ótima de 28ºC a 30ºC, e pH ótimo de 7.2 a 7.4, com tempo de geração de 8 a 12h (LEVETT, 2001). As densidades das culturas não alcançam as obtidas com bactérias convencionais, mas atingem concentrações de 107 a 108 células/mL, com turbidez visível a partir do 4º ou 5º dia (FAINE et al., 1999).

Para o isolamento ou manutenção dos sorovares mais fastidiosos são utilizados suplementos como Tween 80 e adição de BSA (ADLER et al., 1986), ou soro de coelho (FAINE et al., 1999). Muitos meios líquidos contendo soro de coelho já foram descritos por Fletcher, Korthoff, Noguchi e Stuart (TURNER, 1970). Outro meio de cultura que pode ser utilizado é o descrito por Turner, onde a base é o EMJH, com algumas modificações (ALVES et al., 1996).

Além disso, alguns suplementos são adicionados aos meios de cultura para melhorar a taxa de isolamento pois estes componentes ajudam no crescimento de bactérias mais fastidiosas, como o piruvato de sódio (JOHNSON et al., 1973). A enzima superóxido dismutase também pode ajudar no crescimento porque elimina os radicais tóxicos produzidos durante o metabolismo de espiroquetas que se acumulam no meio de cultura (AUSTIN et al., 1981; COX et al., 1990). Também podem ser adicionados ao meio de cultura diferentes combinações de antibióticos que inibem contaminantes presentes na urina ou tecidos utilizados no isolamento inicial de leptospiras (JOHNSON; ROGERS, 1964; MYERS, 1975; ZACARIAS et al., 2008;

MIRAGLIA et al., 2009; CHAKRABORTY et al., 2011).

No entanto, existem poucos estudos sobre o efeito desses suplementos no isolamento e manutenção inicial das estirpes, bem como a influência desses suplementos na viabilidade, motilidade e morfologia das leptospiras.

2.3 ASPECTOS EPIDEMIOLÓGICOS

A leptospirose é uma importante zoonose causada por membros patogênicos do gênero *Leptospira* (KO et al., 2009). Sua distribuição é cosmopolita, porém sua ocorrência é favorecida em regiões de clima tropical e subtropical, onde as elevadas temperaturas e períodos mais chuvosos favorecem a sobrevivência da bactéria (FAINE, 1999). Apesar da sua distribuição mundial, é uma doença com grande impacto na saúde pública nos países em desenvolvimento, onde muitas vezes é sub-diagnosticada (ADLER; DE LA PENA MOCTEZUMA, 2010; BHARTI et al., 2003).

No Brasil, a leptospirose é uma doença endêmica, podendo tornar-se epidêmica nas capitais e regiões metropolitanas durante os períodos com maiores índices pluviométricos, principalmente quando há presença de enchentes, desastres ecológicos (BRASIL, 2019).

A infecção humana resulta principalmente da exposição direta ou indireta à urina de animais infectados. Na área rural, o homem pode adquirir a infecção por contato com bovinos, ovinos, suínos, equinos, cães e diferentes espécies de animais silvestres infectados, ou seja, hospedeiros que estão eliminando a bactéria pela urina e/ou por descargas uterinas e fetos abortados contaminando o ambiente (água, pastagem, alimentos) (Paes et al., 2016). Na área urbana, a população mais acometida são aquelas pessoas que trabalham ou habitam áreas sujeitas a enchentes, em precárias condições de moradia, ausência saneamento de básico, contato com água, lama e esgotos, possivelmente, contaminados por urina infectada de roedores e carnívoros domésticos (BRASIL, 2019; CAMPOS et al., 2011).

Na epidemiologia da leptospirose urbana, o caráter profissional/ocupacional é de grande destaque, abrangendo diferentes categorias profissionais, como por exemplo: agricultores, pecuaristas, trabalhadores de arrozais, trabalhadores de saneamento ambiental, médicos veterinários, biólogos, técnicos de laboratório, manipuladores de produtos de origem animal entre outras profissões

(LEVETT, 2001). Adicionalmente, a infecção pelas leptospiras também pode estar relacionada com atividades recreativas e lazer, embora menos frequente e estreitamente acidental, como banhar-se em rios, riachos, lagos e mananciais que recebem dejetos ou subprodutos de animais ou na prática de atividades esportivas em ambientes possivelmente contaminados (GONÇALVES et al., 2006).

De acordo com sua manifestação clínica, a leptospirose pode ser classificada em dois grupos diferentes: O primeiro é determinado por sorovares adaptados ao hospedeiro, que são menos dependentes das condições ambientais, como topografia ou chuva (sorovar Hardjo em bovinos ou sorovar Canicola em caninos), e geralmente é caracterizada por infecção subclínica, com quadro crônico de portador renal e fonte de infecção para humanos e outros animais. O outro grupo consiste em infecções acidentais causadas por sorovares não adaptados a hospedeiros específicos, e são mais dependentes de fatores ambientais, com transmissão via contato direto ou indireto do humano e/ou animal com a urina do hospedeiro reservatório. Esse último grupo é caracterizado por uma síndrome aguda e grave, como por exemplo, sorovar Pomona em bovinos ou Icterohaemorrhagiae em humanos ou cães (LEVETT, 2001; MACIEL et al., 2008; MARTINS; LILENBAUM, 2013).

A epidemiologia da leptospirose consiste principalmente em identificar os reservatórios presentes nas regiões avaliadas e quais sorovares que eles albergam (BHARTI et al., 2003). Nos centros urbanos e na área rural, os roedores domésticos como *Rattus norvegicus* (ratazana), *Rattus rattus* (rato preto) e *Mus musculus* (camundongo) são considerados os principais reservatórios de sorovares dos sorogrupos Icterohaemorrhagiae e Ballum. A urina e o tecido renal com pH alcalino dos reservatórios são favoráveis para sobrevivência, permitindo a colonização dos túbulos renais e eliminação intermitente da bactéria via urina por toda vida, contaminando água, solo alimentos e além disso, infectar outros animais (FAINE et al., 1999). Outros animais também podem ser hospedeiros de manutenção (reservatórios), bovinos podem abrigar o sorovar Hardjo; os ovinos, Hardjo e Pomona; os suínos, Pomona, Bratislava ou Tarassovi; e os caninos o sorovar Canicola (BARCELLOS et al., 2003; BHARTI et al., 2003).

2.4 MECANISMOS DE PATOGENICIDADE

Na fase inicial da doença, após a entrada das leptospiras no hospedeiro, há uma intensa multiplicação no sangue, fase conhecida por leptospiremia, e posteriormente migram pelos tecidos até atingirem os órgãos-alvo, como rins, pulmões e fígado. As leptospiras não são bactérias intracelulares, mas são capazes de atravessar e residir de forma transitória no interior de células, demonstrando ser um importante mecanismo de evasão do sistema imune e rápida colonização de órgãos do hospedeiro (BAROCCHI et al., 2002; KO et al., 2009). VE-caderina vascular é um componente importante das junções de aderência endotelial, que mantém a integridade vascular onde as leptospiras patogênicas se ligam às células endoteliais que revestem os vasos sanguíneos (EVANGELISTA et al., 2014). No entanto, a VE-caderina detectada por imuno-histoquímica não é o único componente das células endoteliais da microcirculação envolvida na permeabilidade vascular alterada observada na leptospirose. A detecção imuno-histoquímica de glicoproteínas transmembranares endoteliais conhecidas por CD34, também desempenham um papel na adesão celular e mostrou uma capacidade de reduzir a expressão focal nos capilares da microcirculação pulmonar. Além disso, uma expressão reativa aumentada de moléculas de adesão intercelular vascular e receptor do tipo Toll foi identificada nos pulmões de casos de leptospirose humana (ORDÓÑEZ, 2012). Outro mecanismo para translocação epitelial, onde as leptospiras patogênicas, induzem modificações no citoesqueleto de células endoteliais, abrindo um espaço entre elas e permitindo, sua passagem entre as células (MARTINEZLOPEZ et al., 2010).

Leptospiras patogênicas são capazes de evadir dos mecanismos imunes de defesa do hospedeiro, principalmente utilizando estratégias de migração para locais imunoprotegidos, como rins e úvea, e evasão do sistema complemento (FRAGA et al., 2011). Proteínas de membrana externa também são importantes nesta característica de evasão e disseminação da bactéria pelo hospedeiro (CULLEN et al., 2005; HAAKE, MATSUNAGA, 2010). Como a superfície bacteriana é a via de contato entre o patógeno e o hospedeiro, as diferenças nas proteínas de membrana externa podem estar relacionadas aos mecanismos de patogenicidade. As lipoproteínas são abundantes tanto em *L. biflexa*, quanto nas espécies patogênicas. No entanto, mais de 90 lipoproteínas preditas nas espécies patogênicas não têm ortólogos no genoma

da espécie saprofítica, incluindo a lipoproteína majoritária LipL32 (HAAKE et al., 2000), e outras lipoproteínas caracterizadas, como LipL41, LipL36 e LipL45 (CULLEN et al., 2004).

A extensão dos danos nos órgãos dos animais infectados é variável e depende da virulência do sorovar e suscetibilidade do hospedeiro. Uma das principais características das leptospiras patogênicas é a sua replicação e persistência nas células epiteliais dos túbulos renais, onde ocorre a colonização renal (MATHEWS e MONTEITH, 2007), pois é neste local onde as leptospiras ficam protegidas da imunidade humoral (FAINE, 1999). Com a intensa replicação nos túbulos renais as leptospiras passam a serem eliminadas de forma intermitente na urina (leptospirúria) por períodos variáveis (dias a anos). Tal fato explica a existência de portadores renais, fator chave na epidemiologia da leptospirose, onde a transmissão ocorre pela exposição à urina de animais infectados (PLANK; DEAN, 2000).

Outro importante mecanismo para a patogênese da doença é a motilidade das leptospiras que possuem a capacidade de deslocamento em meios altamente viscosos e a localização interna do flagelo periplasmático previne o ataque de anticorpos anti-flagelares (CHARON, GOLDSTEIN, 2002). Estudos mostraram que as proteínas FlaA e FliY que compõem o flagelo são essenciais para a virulência das leptospiras, e a proteína FlaB, essencial para motilidade (LIAO et al., 2009; LAMBERT et al., 2012).

2.5 DIAGNÓSTICO

Tanto na leptospirose animal, quanto na humana, o diagnóstico é considerado difícil, principalmente em locais com recursos limitados, devido à semelhança com as características clínicas de outras doenças, e também devido à escassez de testes de diagnóstico acessíveis e de fácil padronização (FAINE et al., 1999; ADLER; DE LA PENA MOCTEZUMA, 2010).

Uma das dificuldades é que as leptospiras são difíceis de serem coradas pelos métodos tradicionais, como a coloração de Gram, e por terem formato e espessura reduzidos não são visualizadas por microscopia óptica convencional. Portanto, sua visualização somente é possível com a ajuda de técnicas especiais como microscopia de campo escuro ou contraste de fase, coloração por prata ou por conjugados com fluoresceína ou imuno-histoquímica (AVELAR; PEREIRA, 2015).

O exame direto da urina em microscopia de campo escuro consiste na observação da morfologia e motilidade características destas espiroquetas. Entretanto, tem baixa sensibilidade devido à eliminação intermitente da bactéria na urina, além de artefatos de técnica e outras estruturas presentes na amostra que podem ser confundidos com leptospiras (WHO, 2003). Além disso, é necessária rapidez entre a coleta de urina e o exame (15-20 minutos), pois as leptospiras são sensíveis ao pH da urina podendo perder sua motilidade e morfologia que são o objetivo desta técnica, gerando resultados falsos negativos (BOLIN et al., 1989).

O isolamento de *Leptospira* spp. é considerado o diagnóstico definitivo da leptospirose e a partir da obtenção do seu cultivo é possível realizar a identificação do sorovar infectante (ADLER; DE LA PEÑA MOCTEZUMA, 2010). Entretanto, a cultura destas bactérias apresenta várias limitações, como a complexidade dos meios de cultura, a necessidade de semear o material imediatamente após a coleta, o longo tempo de geração, além da dificuldade em obter amostra livre de contaminantes (LILENBAUM, 1996). Mesmo com essas desvantagens é importante realizar o isolamento das leptospiras para posteriormente realizar a identificação e caracterização do novo isolado, através de ferramentas de biologia molecular (PAILHORIÈS et al., 2015; CHIDEROLI et al., 2017).

Os métodos mais utilizados para o diagnóstico da leptospirose são geralmente os testes sorológicos que identificam anticorpos anti-*Leptospira*. No Brasil, os exames utilizados são o ELISA (ensaio imunoenzimático – enzyme linked immuno sorbent assay) para pesquisa de anticorpos IgM e a SAM que é o exame recomendado pela Organização Mundial de Saúde (BRASIL, 2019). Apesar de ser o exame padrão ouro, a SAM é um teste pouco sensível, de difícil padronização e execução, sendo realizado apenas por laboratórios de referência. Outra desvantagem é sua baixa sensibilidade quando o paciente está na fase inicial da doença, já que anticorpos não são detectados nos primeiros dias após a infecção. Além disso, por se tratar de uma metodologia sorovar-específico, os laboratórios necessitam padronizar a técnica e manter culturas vivas de diferentes sorogrupos de *Leptospira* (FAINE et al., 1999; LEVETT, 2003).

Devido aos métodos sorológicos serem limitados, classificando os sorovares apenas em sorogrupo e não conseguir diferenciar os genotipos dentro de espécies diferentes (PICARDEAU, 2013), os métodos moleculares como *Pulse Field Gel Electrophoresis* (PFGE), *Restriction Fragment Length Polymorphism* (RFLP),

Multiple Locus Sequence Typing (MLST), and *Multilocus Variable-Number Tandem-Repeat Analysis* (MLVA) foram introduzidos no diagnóstico, identificação e caracterização de leptospiras (THIERMANN et al., 1985; HERRMANN et al., 1992; PEROLAT et al., 1993; RALPH et al., 1993; MAJED et al., 2005; AHMED et al., 2006).

Outros métodos moleculares mais simples como protocolos de reação em cadeia da polimerase (PCR) têm sido propostos, como uma alternativa a SAM na rotina de diagnóstico da leptospirose. Alguns genes, como por exemplo, *secY, rrs, flaB, LigA, LigB, LipL32, rrl e LA3521*, tem sido utilizados como alvos e amplificados por PCR do liquor e urina de suspeitos de leptospirose (MERIEN et al., 1992; BROWN et al., 1995; KOIZUMI et al., 2004; KOSITANONT et al., 2007). Entretanto, não são eficientes para caracterização e identificação de genotipos, como por exemplo o MLVA, que é rápido, fácil de padronizar e possui custos acessíveis para detectar e identificar sorovares de *Leptospira* (REZASOLTANI et al., 2015), incluindo o genotipo Hardjobovis do sorovar Hardjo (CHIDEROLI et al., 2016). Além disso, alguns estudos demonstraram que os métodos sorológicos e moleculares podem detectar o microrganismo em estágios distintos da doença, e assim, podem fornecer resultados contrários e complementares ao mesmo tempo (BAL et al., 1994; FONSECA et al., 2006; OOTEMAN et al., 2006; KOSITANONT et al., 2007).

Todos os procedimentos diagnósticos apresentam vantagens e desvantagens. Em diagnósticos individuais, o teste sorológico pareado (novo exame após 15 dias) é recomendado para animais e humanos (FAINE, 1999). Portanto, não há uma técnica ideal para todas as situações clínicas. Recomenda-se o uso de uma combinação de testes sorológicos e moleculares ou a SAM pareada que permite uma maior sensibilidade e especificidade no diagnóstico (HASHIMOTO et al., 2017).

2.5.1 Identificação Molecular e Tipificação

Assim como a classificação da leptospirose, seu diagnóstico também não pode ser apenas baseado na identificação e prevalência de sorovares. A identificação e tipificação molecular do agente são essenciais para montar um quadro epidemiológico real da leptospirose em determinada região. Principalmente, quando há casos inconclusivos onde a SAM apresenta títulos baixos para diferentes sorovares e nas reações paradoxais, cujos títulos altos são detectados em sorogrupos que não estão relacionados (LEVETT, 2001; POSTIC et al., 2000).

Por muito tempo, a identificação de novos isolados foi determinada utilizando a classificação sorológica por agrupamento em sorogrupos, entretanto, sua execução é muito laboriosa e de custo elevado, pois necessita da manutenção de um número elevado de estirpes vivas e antissoros (FAINE et al., 1999). Por outro lado, os métodos moleculares são reconhecidamente específicos, já que é possível avaliar seletivamente uma região do genoma exclusiva de determinado microrganismo. As técnicas moleculares têm sido utilizadas em várias áreas, principalmente no diagnóstico de doenças causadas por bactérias de crescimento lento e/ou não-cultiváveis (RIEDIGER, 2007).

Nas últimas décadas, algumas técnicas moleculares foram desenvolvidas e empregadas na tipificação de isolados de leptospiras como, por exemplo, PFGE (HERRMANN et al., 1992), RFLP (ZUERNER et al., 1993), MLVA (MAJED et al., 2005), sequenciamento *secY* (VICTORIA et al., 2008), e MLST (AHMED et al., 2006).

Atualmente, o método MLVA e o sequenciamento do gene *secY* representam uma alternativa prática para diferenciação e identificação de muitos sorovares patogênicos de *Leptospira*, incluindo a diferenciação de dois genotipos de sorovar Hardjo (HAMOND et al., 2015; PAILHORIÈS et al., 2015; CHIDEROLI et al., 2016; UKHOVSKYI et al., 2018; ZARANTONELLI et al., 2018). Além disso, identificar e tipificar novos isolados é importante para entender a epidemiologia da doença na região, bem como desenvolver ferramentas de diagnóstico, vacinas eficazes e estratégias de prevenção para leptospirose (AHMED et al., 2011).

2.6 TRATAMENTO

O tratamento da leptospirose é variável e específico dependendo da fase da doença (FAINE et al., 1999). Em animais de produção não é comum a abordagem curativa e sim o uso dos métodos profiláticos, como vacina e isolamento dos animais doentes. Já nos animais domésticos, principalmente os cães, é rotineiro o tratamento para leptospirose que é muito semelhante ao utilizado no homem (ELLIS, 2015; PAES et al., 2016).

Entre as principais condutas terapêuticas, incluem a hidratação e correção dos distúrbios hidroeletrolíticos, transfusão sanguínea e antibioticoterapia (GUIDUGLI, 2000). Uma variedade de antimicrobianos (doxiciclina, penicilina,

ampicilina, amoxicilina, quinolonas) com diferentes formulações e doses podem ser considerados no tratamento da leptospirose canina (GREENE, 2011). Estes antimicrobianos melhoram os sinais clínicos do paciente, sem, no entanto, eliminar as leptospiras do rim, permanecendo muitas vezes como portador renal e fonte de infecção para o homem e outros animais (OLIVEIRA, 2010).

A penicilina é o principal antimicrobiano de escolha para o tratamento da leptospirose, pois apresenta excelente ação contra as leptospiras, além de ótima difusão pulmonar, entérica, hepática e parênquima renal. No entanto, a penicilina não consegue penetrar nas células dos túbulos renais nas quais a bactéria persiste e se multiplica, levando o animal ao estado de portado renal com leptospirúria prolongada (PAES et al., 2016).

Acredita-se que a colonização por leptospiras patogênicas dos túbulos renais proximais de hospedeiros mamíferos acarretam na formação de agregados celulares e biofilme, levando à persistência da infecção e estado de portador renal. Em um estudo com camundongos C57BL / 6, foi observado a presença de leptospiras bioluminescentes demonstrando que a colonização renal por leptospiras fornece escape furtivo do mecanismo de defesa imunológico e de antimicrobianos (RATET et al., 2014). Ratet e colaboradores (2014), concluiram que o tratamento com penicilina, ciprofloxacina e doxiciclina falhou em eliminar todas as leptospiras dos túbulos em estágio crônico. Da mesma forma, Kumar e colaboradores (2016) demonstraram *in vitro* resistência a esses antimicrobianos devido a formação de biofilme. Esses resultados estão de acordo com os dados da literatura, que relatam que a penicilina, ampicilina e amoxicilina são eficientes apenas no estágio inicial da infecção (PAES et al., 2016).

Portanto como estratégia terapêutica, após a terapia na fase de leptospiremia, recomenda-se o uso de outros antimicrobianos com o intuito de eliminar leptospiras presentes nos túbulos renais. Com base no tratamento instituído para o homem, nessa fase pós leptospiremia, a doxiciclina tem sido o fármaco de eleição para os cães e outros animais por um período de duas semanas por apresentar elevada concentração terapêutica renal (WOHL, 1996; TAVARES, 2014).

Apesar da doxiciclina ser considerada como uma opção de antimicrobiano para a eliminação das bactérias dos túbulos renais, a estreptomicina é considerada muito mais efetiva (PAES et al., 2016). Entretanto, por ser um antimicrobiano da classe dos aminoglicosídeos, seu uso deve ser restringido quando

o animal apresentar sinais de azotemia e insuficiência renal aguda devido a sua nefrotoxicidade (OLIVEIRA et al., 2006).

Dados sobre suscetibilidade antimicrobiana de isolados de *Leptospira* ainda são escassos, principalmente devido a sua dificuldade de isolamento e crescimento lento a partir de amostras biológicas. Uma técnica de microdiluição foi desenvolvida para *Leptospira* para avaliação da suscetibilidade a antibióticos (MURRAY; HOSPENTHAL, 2004) e tem sido utilizada para a avaliação da CIM para cepas isoladas de humanos, ratos, cães e suínos. Esses estudos mostraram que as leptospiras são suscetíveis aos antimicrobianos mais utilizados no tratamento da leptospirose, mas é resistente ao trimetoprim, sulfametoxazol, neomicina, polimixina. Além disso, indicam um aumento da CIM para tetraciclina e doxiciclina (CHAKRABORTY et al., 2010; SUEPAUL et al., 2015; BENACER et al., 2017; LIEGEON et al., 2018). Independente do protocolo para tratamento da leptospirose é fundamental seguir três etapas: inativação do agente, restabelecimento e manutenção das funções renal e hepática. Quando a escolha dos antimicrobianos não é realizada de forma adequada, os animais aparentemente recuperados podem tornar-se portadores convalescentes e desenvolver nefrite intersticial crônica (PAES et al., 2016).

Combater o estado portador renal crônico é atualmente o principal objetivo dos estudos sobre tratamento de leptospirose. Apesar de estudos de susceptibilidade indicarem que as leptospiras são sensíveis a maioria dos antimicrobianos é necessário ficar alerta, pois no contexto atual de bactérias multiresistentes e doenças crônicas, o monitoramento de cepas de *Leptospira* em animais domésticos e de produção pode ajudar a detectar o surgimento de resistência antimicrobiana (LIEGEON et al., 2018). Assim, o uso de ferramentas de bioinformática pode auxiliar na busca de novos alvos drogáveis com capacidade de eliminar as bactérias dos túbulos renais e evitar o estado de portador renal dos animais.

2.7 Controle e Prevenção

As medidas de controle e prevenção da leptospirose estão relacionadas ao controle da população de roedores, à melhoria das condições higiênico-sanitárias da população, educação em saúde humana e animal e cuidados

com o meio ambiente (BRASIL, 2019). Entretanto, como estas são medidas fundamentais e de difícil implementação, o desenvolvimento de uma vacina é primordial para um melhor controle e prevenção da doença. As vacinas contra a leptospirose disponíveis atualmente no mercado são veterinárias e compostas de bactérias inativadas (bacterinas), contudo, pesquisas buscam também vacinas recombinantes de proteínas de membrana de espécies patogênicas (GAMBERINI et al., 2005; KO; GOARANT; PICARDEAU, 2009; ADLER; DE LA PENA MOCTEZUMA, 2010).

Esses antígenos, principalmente o LPS, estimulam uma resposta timo independente, ou seja, baixa eficiência na proteção a longo prazo contra a infecção, o que torna necessário seu reforço semestral ou a cada 3 meses se a região for endêmica (BOLIN et al., 1989). Além disso, as vacinas comerciais são sorovares específicas, portanto, não promovem proteção cruzada contra leptospiras de sorovares diferentes (FAINE et al., 1999; LEVETT, 2001). Por essas características é cada vez maior a busca por vacinas contra um maior número de sorovares patogênicos do gênero *Leptospira* (ADLER, 2015).

Estudos buscam encontrar proteínas relacionadas à patogenicidade ou virulência, e que tenham um amplo espectro de conservação dentre os principais sorovares patogênicos ao homem e aos animais (HAAKE et al., 1999; GAMBERINI et al., 2005; YANG et al., 2006; RISTOW et al., 2007; FAISAL et al., 2008; FENG et al., 2009; CAO et al., 2011; RAJA; NATARAJASEENIVASAN, 2015; DELLAGOSTIN et al., 2017; GRASSMANN et al., 2017a). A identificação dessas proteínas poderá servir de base para o desenvolvimento de uma vacina que seja eficiente contra a leptospirose e além disso, confira proteção a longo prazo.

Vários grupos de pesquisa usaram a abordagem clássica para a identificação de alvos proteicos na tentativa de criar vacinas recombinantes, e os resultados foram variados (DELLAGOSTIN et al., 2011). Os alvos mais promissores até o momento são as proteínas do tipo imunoglobulina (Lig). Recentemente, foi testada uma vacina de subunidade LigB, a qual protegia hamsters contra leptospirose e induzia imunidade (CONRAD et al., 2017). A proteção conferida pela LigA, no modelo de hamster, tem sido bastante reproduzida por diferentes grupos em todo o mundo, entretanto ainda precisa ser testada em outros animais, pois a imunidade prolongada não foi tão evidente em sobreviventes vacinados com LigA (KOIZUMI; WATANABE, 2004; COUTINHO et al., 2011). Além disso, outra grande desvantagem

é que a proteína ligA está presente em apenas três *Leptospira* spp patogênicas, o que pode limitar sua capacidade de induzir imunidade cruzada (FOUTS et al., 2016).

É importante observar também que LipL32, uma lipoproteína imunodominante das leptospiras, foi extensivamente investigada para ser candidata a vacina, mas teve resultados inconclusivos com relação a eficácia variando de 12 a 87% (BRANGER et al., 2005; SEIXAS et al., 2007; GRASSMANN et al., 2012). Outra lipoproteína putativa, LemA, foi identificada através da VR mas sua proteção foi parcial e induzida usando a estratégia de reforço em curtos períodos (HARTWIG et al., 2011). Além disso, a proteína da membrana externa putativa (OMP) OmpL37, recentemente caracterizada, foi considerada um dos antígenos mais promissores, entretanto, não induziu proteção no modelo de hamster (OLIVEIRA et al., 2015).

A falta de terapêutica e vacina adequadas contra a leptospirose está aumentando a carga desta doença em todo o mundo. A vacinação contra a leptospirose em populações humanas pode ser a abordagem mais adequada para o controle da doença. Porém, devido a seus efeitos adversos, imunidade a curto prazo e insuficiência na indução da proteção cruzada, eles não foram implementados globalmente igual a utilizada em animais. Na China, uma vacina contra leptospirose foi desenvolvida com sucesso em 1958, a qual tem sido usada para imunização de populações de risco de regiões epidêmicas até agora. Com os avanços da biotecnologia, a produção da vacina melhorou gradualmente (YAN et al., 2006). Em 1958– 1962, uma vacina inativada trivalente contendo sorogrupos Icterohaemorrhagiae, Autumnalis e Pyrogenes foi usada para vacinação em larga escala em áreas epidêmicas e diminuiu muito a morbidade (ZHANG et al., 2012)

O *status* atual do desenvolvimento da vacina para leptospirose ainda mostra falhas, indicando a necessidade de buscar novos alvos vacinais com uso da VR (RAPPUOLI, 2001), mas para isso exigirá uma reavaliação de mais genomas de *Leptospira* (GRASSMANN et al., 2107b). Atualmente, existe uma grande quantidade de dados genômicos e proteômicos e uma variedade de ferramentas de bioinformática disponíveis, assim, a análise *in silico* dos genomas leptospirais deve ser extensivamente explorada para aumentar a probabilidade dos antígenos preditos serem de fato proteínas expostas à superfície e, portanto, potenciais alvos vacinais (DELLAGOSTIN et al., 2017).

2.8 ESTADO DA ARTE: GENOMA DA *LEPTOSPIRA* SPP

2.8.1 Conceitos Sobre Genoma e Genômica

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Os estudos genômicos são vastos e podem incluir a organização, função e evolução do material genético no nível de todo o genoma. O estudo sistemático da informação contida no genoma pode ser aplicado para resolver questões de biologia, medicina e biotecnologia.

Nas últimas décadas, o desenvolvimento de tecnologias de sequenciamento de próxima geração (NGS) e ferramentas de bioinformática levou à caracterização de muitos genomas procarióticos em um período muito curto de tempo. Em comparação aos genomas eucarióticos, os genomas microbianos demonstram variações na estrutura e na densidade de conteúdo (KOONIN; WOLF, 2008). A pressão biológica e a adoção de diversos ambientes podem promover alterações nos genomas das bactérias, por exemplo, os microrganismos de vida livre provavelmente necessitem de um arsenal de adaptação mais extenso, refletido por um genoma maior, pois podem encontrar variadas situações críticas para a sua sobrevivência quando comparada aos microrganismos patogênicos (BULACH et al., 2006). Por isso, múltiplos *replicons* de DNA (mais de um cromossomo) podem existir em bactérias e algumas bactérias contêm cópias únicas ou múltiplas de plasmídeos essenciais ou não essenciais (NISHIDA, 2012).

Recentemente, surgiu uma subárea dentro do estudo “Genômico” chamada de genômica comparativa, ou seja, uma abordagem abrangente que compara dois ou mais genomas para descobrir as semelhanças e diferenças entre eles e também estudar individualmente a informação genética e biológica de cada um (TOUCHMAN, 2010). Além disso, os dados obtidos desta comparação fornecem informações adicionais sobre a evolução microbiana, inclusive sobre fenômenos como a transferência de genes (JENSEN et al., 1999; USSERY et al., 2008). A chegada de tecnologias de NGS associadas às ferramentas de bioinformática aprimorou a ciência genômica e tornaram possível sequenciar o genoma de microrganismos de diversos habitats. Consequentemente, o número de sequências de genomas completos depositados em bancos públicos de dados está aumentando exponencialmente (PAGANI et al., 2012).

Além disso, a análise do genoma microbiano pode colaborar com

estudos de fenômenos biológicos e com aplicações mais amplas em biotecnologia. E com uso combinado de ferramentas de bioinformática essa análise pode ser aplicada em novas abordagens para o tratamento e controle de organismos patogênicos (METZKER, 2005; FORDE; O'TOOLE, 2013).

A disponibilidade de sequências genômicas completas (conjuntos completos de genes / proteínas) permite identificar conjuntos de genes ortólogos, ou seja, genes originados do mesmo gene pertencente ao ancestral comum dos genomas comparados. Mais importante, a partir das informações obtidas das análises comparativas é possível determinar não apenas quais genes estão presentes em determinado genoma, mas também quais estão ausentes (GRANT et al., 2012; KOONIN & WOLF, 2008).

A genômica comparativa ajuda a encontrar e entender as características genômicas comuns (básicas) e únicas (especiais) em espécies intimamente relacionadas. Portanto, colabora na busca do conhecimento sobre os mecanismos relacionados à evolução, adaptação ao hospedeiro e ao meio ambiente e, mais importante, à patogenicidade (PALLEN & WREN, 2007). Diferentes organismos podem compartilhar algumas características comuns e, por análises comparativas, as diferentes espécies podem fornecer informações genômicas sobre vias metabólicas, genes de resistência e/ou processos biológicos essenciais comum. Por outro lado, a comparação de espécies intimamente relacionadas pode revelar as origens de características e eventos adaptativos (genes de virulência, ilhas de patogenicidade, dentre outros.) (DONATI; RAPPUOLI, 2013).

Consequentemente, com o estudo genômico comparativo de espécies bacterianas descobriu-se uma diversidade substancial de informações genéticas, resultando na criação de uma nova área dentro da genômica comparativa conhecida como "pangenômica", abordada pela primeira vez por Tettelin e colaboradores (2005). O pan-genoma é a descrição da coleção de genes encontrados no *pool* genético dos genomas comparados de várias estirpes de uma mesma espécie de determinado microrganismo. O conceito pan-genoma também pode ser definido como "o repertório global de genes de um grupo intimamente relacionados (de preferência da mesma espécie)" e inclui o *core* genoma (genes comuns a todas as estirpes), o genoma acessório (genes presentes em algumas estirpes) e o genoma único (genes específicos de uma estirpe), sendo os dois últimos chamados de genes acessórios (MEDINI et al., 2005).

2.8.2 Genoma da *Leptospira* spp.

A espécie patogênica *L. interrogans Lai* estirpe 56601 foi a primeira leptospira a ter seu genoma sequenciado e depositado no banco de dados GenBank, e as informações obtidas das análises *in silico* revelaram características fisiológicas únicas e os fatores de virulência dessa cepa (REN et al., 2003). Posteriormente, os genomas de várias outras estirpes patogênicas e não patogênicas foram submetidos ao NGS para investigar os mecanismos de patogênese da *Leptospira* (FOUTS et al., 2016). A espécie patogênica, *L. borgpetersenii* teve o seu genoma sequenciado, devido sua importância em rebanhos de bovinos e suínos causando grande impacto econômico na pecuária (BULACH et al., 2006). Além das espécies patogênicas, a espécie saprófita, *L. biflexa* foi sequenciada para ajudar a compreender os mecanismos de patogenicidade comparando o genoma de espécies patogênicas e saprofíticas (PICARDEAU et al., 2008).

A partir desses estudos, foi determinado que o genoma das leptospiras é composto por dois cromossomos circulares, porém foi encontrado um terceiro *replicon* denominado P74 na espécie saprofítica *L. biflexa* e ausente nas espécies patogênicas. Sugere-se que este *replicon* seja essencial para esta espécie de vida livre, pois possui genes que participam do metabolismo basal e que são encontrados no cromossomo I das leptospiras patogênicas. Além disso, a comparação dos genomas colaborou na identificação de 1431 genes únicos das espécies patogênicas, ou seja, sem genes ortológos na *L. biflexa* (PICARDEAU et al., 2008). Os genomas de maior tamanho (~4,6 Mb) são encontrados nas estirpes de *L. interrogans* comparados aos genomas de *L. borgpetersenii* e *L. biflexa* (~3,9 Mb). A comparação destes genomas foi realizada por Bulach e colaboradores (2006), que observaram a perda de genes relacionados com a sobrevivência fora do hospedeiro, limitando a transmissão de sorovares da *L. borgpetersenii* apenas pelo contato direto com animais infectados. Por outro lado, o maior genoma de *L. interrogans* provavelmente é consequência da presença de genes relacionados a sua sobrevivência tanto no meio ambiente quanto nos variados hospedeiros (FOUTS et al., 2016).

Em relação aos genes rRNA, a maioria das bactérias possui estes genes organizados em *operon*, entretanto, descobriu-se que nas leptospiras estes genes estão distribuídos ao longo do cromossomo CI. Ambos os genomas revelaram

número muito pequeno de cópias dos genes rrf, rrs e rrl, que codificam as subunidades ribossômicas 5S, 16S e 23S, respectivamente (NASCIMENTO et al., 2004b). Esse fato pode explicar parcialmente o crescimento fastidioso das leptospiras (REN et al., 2003).

Um estudo genômico comparativo mais recente, realizado por Xu e colaboradores (2016), demonstrou que as diversas populações globais de espécies patogênicas de *Leptospira* possuíam genomas grandes e variados. Por apresentar um pan-genoma aberto pode indicar uma alta variabilidade genômica entre as espécies estudadas o que pode ser atribuído a eventos aleatórios de ganho e perda de genes (ROULI et al., 2015). Xu e colaboradores (2016), observaram que mais genes foram perdidos do que ganhos antes da separação em grupos patogênicos e intermediários, contudo, mais genes foram ganhos do que perdidos na evolução individual de cada espécie patogênica. Este mesmo grupo de pesquisa também analisou a filogenômica comparativa e sugeriram que os eventos horizontais de transferência e duplicação de genes facilitaram a aquisição gradual de fatores de virulência (Xu et al., 2016).

Outro dado importante obtido dos estudos genômicos é a ocorrência de um grande número de elementos genéticos móveis (IS – *insertion sequences*). São encontrados mais de 120 IS no genoma de *L. borgpetersenii*, 26 na *L. interrogans* sorovar Copenhageni, 57 no sorovar Lai e 9 na *L. biflexa*. Estes IS são indicativos de plasticidade genômica e sugerem uma participação no processo de diferenciação das espécies do gênero *Leptospira* (PICARDEAU et al., 2008). Foi proposto que os IS participaram na redução genômica da *L. borgpetersenii*, que é 16% menor que da espécie *L. interrogans* (BULACH et al., 2006).

Assim, vários estudos dos genes que codificam proteínas imunogênicas e virulentas estão sendo realizados para uma melhor compreensão da patogenia da leptospirose tanto para o desenvolvimento de vacinas quanto para a descoberta de novos alvos drogáveis (VIEIRA, 2008; HASHIMOTO, 2012).

2.9 Docking Molecular e Vacinologia Reversa

O processo de identificação de um alvo protéico, síntese de um composto ativo com características adequadas, como toxicidade mínima, alta biodisponibilidade, síntese econômica, e finalmente o desenvolvimento para introdução no mercado de um novo medicamento é um experimento demorado,

extremamente complexo e arriscado (MUNTHA, 2016). Inicialmente, é identificado um alvo que desempenha um papel fundamental na patologia de uma doença e seja vital para o metabolismo e sobrevivência do patógeno relacionado a doença. Uma vez estabelecido a conexão entre o alvo e a doença/ microrganismo, o próximo passo é identificar possíveis compostos que possam interromper ou reverter o progresso da doença ao se ligar no alvo selecionado.

A triagem para identificar novas moléculas é um processo no qual normalmente um grande número de compostos de produtos naturais e bancos de dados on-line são examinados quanto à atividade biológica em ensaios de alto rendimento. Essa etapa do processo de descoberta de medicamentos é muito importante e exige a manutenção de grandes bibliotecas moleculares e a realização de milhares de ensaios, que é a parte com mais desvantagens para as empresas farmacêuticas e também de maior custo. Do ponto de vista acadêmico, realizar triagem experimental tradicional de alto rendimento (TTAR) é caro, demorado e inviável (ALVAREZ, 2004).

Com o sequenciamento de próxima geração houve um aumento de sequenciamento e montagem de genomas, principalmente de procariotos, e resultou em um número crescente de novos alvos terapêuticos para a descoberta de medicamentos. Ao mesmo tempo, foram desenvolvidas técnicas de purificação de proteínas, cristalografia e ressonância magnética nuclear que contribuíram para a definição de detalhes estruturais de proteínas e de complexos proteína-ligante (JORGENSEN, 2004). Esses avanços permitem que as estratégias de bioinformática possam contribuir na descoberta de novos medicamentos (KITCHEN et al., 2004), utilizando por exemplo, técnicas de triagem virtual (TV) para otimização da busca pela melhor ligação proteína-ligante. Comparado com a HTS, a TV é uma abordagem de descoberta de drogas mais direta e rápida com a vantagem de ser uma triagem de baixo custo e mais eficaz (MOITESSIER et al., 2008).

A abordagem de docking molecular consiste em modelar, a nível atômico, a interação entre uma molécula pequena (ligante) e uma proteína, o que permite caracterizar o comportamento desses ligantes no sitio de ligação com as proteínas alvos (MCCONKEY et al., 2002). O processo de docking é desenvolvido em várias etapas: preparação da proteína e identificação do sitio de ligação, previsão da conformação do ligante, sua posição e orientação dentro desses locais (geralmente chamados de pose) e avaliação da afinidade de ligação (MENG et al., 2011).

Normalmente, o ligante estabiliza um subconjunto de várias conformações possíveis com a proteína alvo, e assim, gerando um equilíbrio com um gasto energético mínimo. Além disso, quando nenhuma estrutura cristalográfica adequada estiver disponível para um alvo molecular específico (isto é, estruturas com locais de ligação inacessíveis ou mal definidos), o docking molecular pode ser aplicado para gerar um conjunto de estruturas mais convenientes para acoplamento (SINGH; WARSHEL, 2010).

Outra ferramenta de bioinformática com crescente adesão dos pesquisadores é a vacinologia reversa (VR), que facilita a descoberta de alvos vacinais para diversas doenças. A abordagem da VR foi a primeira ferrramenta que utilizou a aplicação de tecnologias genômicas e representou uma grande revolução no processo de descoberta de novas vacinas (RAPPUOLI, 2001).

Com a possibilidade de determinar uma lista de proteínas antigênica usando a VR, os pesquisadores puderam identificar alvos protetores e buscar por vacinas mais eficazes contra diversos patógenos quando as abordagens tradicionais falharam. O uso da bioinformática iniciou várias mudanças que afetaram todo o processo de desenvolvimento de uma vacina, sendo a principal mudança relacionada à definição de um conjunto triagem de alto rendimento para diminuir a necessidade de testes laboratoriais em animais que são muito onerosos e trabalhosos (DONATI; RAPPUOLI, 2013).

Essa tecnologia pode substituir os métodos tradicionais de preparo de vacinas, onde elimina-se a necessidade de que os patógenos sejam inicialmente cultivados, ou modificados em suas características de virulência, ou seus fragmentos sejam isolados e purificados previamente (DONATI; RAPPUOLI, 2013). A primeira etapa inicia-se com o sequenciamento do genoma do microrganismo responsável por uma doença, análise de suas proteínas com base nas características hidrofóbicas ou hidrofílicas, determinando-se a posição provável das proteínas dentro do microorganismo utilizando a bioinformática. Posteriormente, é avaliada sua capacidade teórica de produzir resposta imune e finalmente os peptídeos selecionados podem, então, ser sintetizados ou expressos em vetores para a comprovação de sua real capacidade de induzir imunidade em animais (ADU-BOBIE et al., 2003).

O gênero *Leptospira* é bastante complexo, entretanto existe uma grande quantidade de genomas depositados em bancos de dados públicos, contribuindo para

a realização de estudos sobre a prevenção, controle e tratamento utilizando ferramentas *in silico* para buscar novos alvos vacinais e novos medicamentos (SRITRAKULA et al., 2017).

REFERÊNCIAS

ADLER, B.; FAINE, S.; CHRISTOPHER, W.L.; CHAPPEL, R.J. Development of an improved selective medium for isolation of leptospires from clinical material. **Veterinary Microbiology**, v. 12, p. 377-381, 1986. Disponível em: https://doi.org/10.1016/0378-1135(86)90087-8

ADLER, B.; DE LA PEÑA MOCTEZUMA, A. *Leptospira* and leptospirosis. **Veterinary Microbiology**, v. 140, n. 3-4, p. 287–296, 2010. Disponível em: https://doi.org/10.1016/j.vetmic.2009.03.012

ADLER B. Vaccines against leptospirosis. *IN*: **Current topics in microbiology and immunology,** Verlag Berlin Heidelberg: Editora Springer, p. 251–272, 2015.

AHMED, N.; DEVI, S.M.; VALVERDE, M.L.; VIJAYACHARI, P.; MACHANG'U, R.S.; ELLIS, W.A., et al. Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species. **Ann. Clin. Microbiol. Antimicrob**. v. 5, p.28, 2006. Disponível em: https://doi.org/ 10.1186/1476-0711-5-28

AHMED, A., THAIPADUNGPANIT, J., BOONSILP, S., WUTHIEKANUN, V., NALAM, K., SPRATT, B. G., et al. Comparison of two multilocus sequence based genotyping schemes for Leptospira species. **PLoS Negl**. Trop. Dis. v. 5, p. e1374, 2011. Disponível em: doi:10.1371/journal.pntd.0001374.

ALVAREZ, J.C. High-throughput docking as a source of novel drug leads. **Current Opinion in Chemical Biology**. v. 8, n. 4, p. 365-370, 2004. Disponível em: doi:10.1016/j. cbpa.2004.05.001

ALVES, C.J., VASCONCELOS, S.A., CAMARGO, C.R.A., MORAES Z.M. Influência de fatores ambientais sobre a proporção de caprinos soro-reatores para a leptospirose em cinco centros de criação do Estado da Paraíba, Brasil. Arqs Inst. Biológico, São Paulo, 63(2):11-18, 1996.

AUSTIN, F.E., BARBIERI, J.T., CORIN, R.E., GRIGAS, K.E., COX, C.D. Distribution of superoxide dismutase, catalase, and peroxidase activities among *Treponema pallidum* and other spirochetes. **Infect. Immun**. v. 33, p. 372–379, 1981.

AVELAR, K.E.S; PEREIRA, M.M. Espiroquetídeos. *In:* TRABULSI, L.R; ALTERTHUM, F**. Microbiologia,** 6ª. ed Editora Atheneu, São Paulo, 2015.

BAL, A.E. et al. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. **Journal of Clinical Microbiology**, Washington D. C., v. 32, n. 8, p. 1894-1898, 1994.

BARCELLOS, C., LAMMERHIRT, C.B., DE ALMEIDA, M.A., et al. Spatial distribution of leptospirosis in Rio Grande do Sul, Brazil: recovering the ecology of ecological studies. **Cadernos de Saúde Pública**, v. 19, p. 1283-1292, 2003. Disponível em:

http://dx.doi.org/10.1590/S0102-311X2003000500007.

BAROCCHI, M.A.; KO, A.I.; REIS, M.G.; MCDONALD, K.L.; RILEY, L.W. Rapid translocation of polarized mdck cell monolayers by leptospira interrogans, an invasive but nonintracellular pathogen. **Infect Immun**, v. 70, n. 12, p. 6926-6932, 2002. Disponível em: http://dx.doi.org/10.1128/iai.70.12.6926-6932.2002.

BENACER, D., ZAIN, S.N.M., OOI, P.T., THONG, K.L. Antimicrobial susceptibility of Leptospira spp. isolated from environmental, human and animal sources in Malaysia. Indian J Med Microbiol v. 35, p. 124–128, 2017. (10.4103/ijmm.IJMM_15_458)

BHARTI, A.R., NALLY, J.E., RICALDI, J.N., MATTHIAS, M.A., DIAZ, M.M., et al. Leptospirosis: a zoonotic disease of global importance. **Lancet Infection Disease**, v. 3, p. 757-771, 2003. Disponível em: 10.1016/s1473-3099(03)00830-2

BOLIN, C.A, THIERMANN, A.B, HANDSAKER, A.L, FOLEY, J.W. Effect of vaccination with a pentavalent leptospiral vaccine on Leptospira interrogans serovar hardjo type hardjo-bovis infection of pregnant cattle. **Am J Vet Res**. v. 50, p. 161–165, 1989.

BRANGER, C., CHATRENET, B., GAUVRIT, A., AVIAT, F., AUBERT, A., BACH, J.M, et al. Protection against Leptospira interrogans sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. **Infect Immun**, v. 73, n. 7, p. 4062–4069, 2005. Disponível em: https://doi.org/10.1128/IAI.73.7.4062-4069.2005

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Coordenação-Geral de Desenvolvimento da Epidemiologia em Serviços. **Guia de Vigilância em Saúde: volume único [recurso eletrônico]** – 3ª. ed. – Brasília: Ministério da Saúde, 2019.

BROWN, P.D. et al. Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. **J. Med. Microbiol**., v. 43, n. 2, p. 110-114, 1995. Disponível em: https://doi.org/10.1099/00222615-43-2-110

BULACH, D.M., ZUERNER, R.L., WILSON, P., SEEMANN, T., MCGRATH, A., CULLEN, P.A, et al. Genome reduction in Leptospira borgpetersenii reflects limited transmission potential. **Proc Natl Acad Sci U S A**, v.103, n. 39, p. 14560–5, 2006. Disponível em: 10.1073/pnas.0603979103

CAIMI, K., REPETTO, S.A., VARNI, V., RUYBAL, P. Leptospira species molecular epidemiology in the genomic era**. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis**. v. 54, p. 478–85, 2017. https://doi.org/10.1016/j.meegid.2017.08.013

CAMPOS, H. et al. Leptospirose saúde ambiental, saneamento básico e urbanização. Revista de Trabalhos Acadêmicos, América do Norte, 2, jun. 2011

CAO, Y., FAISAL, S.M., YAN, W., CHANG, Y.C., MCDONOUGH, S.P., ZHANG, N., AKEY, B.L., CHANG, Y.F. Evaluation of novel fusion proteins derived from extracellular matrix binding domains of LigB as vaccine candidates against leptospirosis in a hamster model. **Vaccine**. v. 29, p. 7379–7386, 2011. Disponível em: https://doi.org/10.1016/j.vaccine.2011.07.070

CERQUEIRA, G.M.; PICARDEAU, M. A century of Leptospira strain typing. **Infection and Genetic Evolution**, v. 9, n. 5, p. 760-768, 2009. Disponível em: https://doi.org/10.1016/j.meegid.2009.06.009

CHAKRABORTY, A., MIYAHARA, S., VILLANUEVA, S.Y. A. M., GLORIANI, N.G, YOSHIDA, S. In Vitro Sensitivity and Resistance of 46 Leptospira Strains Isolated from Rats in the Philippines to 14 Antimicrobial Agents. Antimicrobial Agents and Chemotherapy, v. 54, n. 12, p. 5403-5405, 2010. DOI: 10.1128/AAC.00973-10

CHAKRABORTY, A., MIYAHARA, S., VILLANUEVA, S. Y. A. M., SAITO, M., GLORIANI, N. G., YOSHIDA, S. I. A novel combination of selective agents for isolation of Leptospira species. **Microbiol. Immunol**. v. 55, p. 494–501, 2011. Disponível em: https://doi.org/10.1111/j.1348-0421.2011.00347.x

CHARON, N. W.; GOLDSTEIN, S. F. Genetics of motility and chemotaxis of a fascinating group of bacteria: The spirochetes. **Annu Rev Genet**, v. 36, p. 47-73, 2002. Disponível em: https://doi.org/10.1146/annurev.genet.36.041602.134359

CHIDEROLI, R.T., PEREIRA, U.P., NAKAMURA, A.Y., ALFIERI, A.A., ALFIERI A.F., FREITAS, J.C. Isolation and molecular characterization of *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis in the urine of naturally infected cattle in Brazil. **Genet. Mol. Res**. v. 15, p. 1–7, 2016. Disponível em: https://doi.org/10.4238/gmr.15018473

CHIDEROLI, R.T., GONÇALVES, D.D., SUPHORONSKI, S.A., ALFIERI, A.F., ALFIERI, A.A., DE OLIVEIRA, A.G., DE FREITAS, J.C., PEREIRA, U.P. Culture Strategies for Isolation of Fastidious *Leptospira* Serovar Hardjo and Molecular Differentiation of Genotypes Hardjobovis and Hardjoprajitno. **Front Microbiol**. v. 2, n.8, p. 2155, 2107. Disponível em: https://doi.org/10.3389/fmicb.2017.02155.

CONRAD, N.L., CRUZ, MCBRIDE, F.W., SOUZA, J.D., SILVEIRA, M.M., FELIX, S., MENDONCA, K.S., et al. LigB subunit vaccine confers sterile immunity against challenge in the hamster model of leptospirosis. **PLoS Negl Trop Dis**, v. 11, n. 3, 2017. https://doi.org/10.1371/journal.pntd.0005441

COUTINHO, M.L., CHOY, H.A., KELLEY, M.M., MATSUNAGA, J., BABBITT, J.T., LEWIS, M.S., et al. A LigA three-domain region protects hamsters from lethal infection by Leptospira interrogans. **PLoS Negl Trop Dis**, v. 5, n. 12, 2011. https://doi.org/10.1371/journal.pntd.0001422

COX, D.L., RILEY, B., CHANG, P., SAYAHTAHERI, S., TASSELL, S., HEVELONE, J. Effects of molecular oxygen, oxidation-reduction potential, and antioxidants upon in vitro replication of Treponema pallidum subsp. pallidum. **Appl. Environ. Microbiol.** v. 56, p. 3063–3072, 1990.

CULLEN, P.A.; HAAKE, D.A.; ADLER, B. Outer membrane proteins of pathogenic spirochetes. **FEMS Microbiol. Rev**., v. 28, p. 291-318, 2004. doi:10.1016/j.femsre.2003.10.004

CULLEN, P.A.; XU, X.; MATSUNAGA, J.; SANCHEZ, Y.; KO, A. I.; HAAKE, D.A.; ADLER, B. Surfaceome of Leptospira spp. **Infect Immun**, v.73, n.8, p.4853-4863, 2005. Disponível em: https://doi.org/10.1128/IAI.73.8.4853-4863.2005

DELLAGOSTIN, O.A., GRASSMANN, A.A., HARTWIG, D.D., FELIX, S.R., DA SILVA, E.F., MCBRIDE, A.J. Recombinant vaccines against leptospirosis. **Hum. Vaccine**. v. 7, p. 1215–1224, 2011. Disponível em: https://doi.org/10.4161/hv.7.11.17944

DELLAGOSTIN, O.A., GRASSMANN, A.A., RIZZI, C., SCHUCH, R.A., JORGE, S., et al. Reverse Vaccinology: An Approach for Identifying Leptospiral Vaccine Candidates. **Int J Mol Sci**. v. 18, n. 1, p. 158, 2017. Disponível em: https://doi.org/10.3390/ijms18010158.

DONATI, C.; RAPPUOLI, R. Reverse vaccinology in the 21st century: improvements over the original design. **Ann N Y Acad Sci**, v. 1285, p. 115-132, 2013. Disponível em: https://doi.org/10.1111/nyas.12046

ELLIS, W.A.; THIERMANN A. B. Isolation of leptospires from the genital tracts of Iowa cows. **Am. J. Vet. Res**. v.47, p.1694–1696, 1986.

ELLIS, W.A. Animal leptospirosis. *In:* **Current Topics in Microbiology and Immunology**. ADLER, B. Verlag Berlin Heidelberg: Editora Springer, p. 99–137, 2015.

EVANGELISTA K, FRANCO R, SCHWAB A, COBURN J. Leptospira interrogans binds to cadherins. PLoS Negl Trop Dis. 2014;8:e2672

FAINE, S., ADLER B., BOLIN C. & PEROLAT P. **Leptospira and Leptospirosis**. 2nd ed. 690 Medi Science, Melbourne. 272p, 1999.

FAISAL, S.M.; YAN, W.; CHEN, C.S.; PALANIAPPAN, R.U.; MCDONOUGH, S.P.; CHANG, Y.F. Evaluation of protective immunity of Leptospira immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. **Vaccine**, v. 26, n. 2, p. 277-287, 2008. https://doi.org/10.1016/j.vaccine.2007.10.029

FENG, C.Y.; LI, Q.T.; ZHANG X.Y.; DONG K.; HU B.Y.; GUO X.K. Immune strategies using single-component LipL32 and multi-component recombinant LipL32–41-OmpL1 vaccines against leptospira. **Braz. J. Med. Biol. Res**. v. 42, p. 796–803, 2009. Disponível em: https://doi.org/10.1590/S0100-879X2009005000013

FLINT, S.H.; LIARDET, D.M. Isolation of *Leptospira interrogans* serovar Hardjo from bovine urine. **N. Z. Vet. J**. v. 28, n. 3, p. 55, 1980. Disponível em: https://doi.org/10.1080/00480169.1980.34693

FONSECA, C. A. et al. Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis. **Tropical Medicine and International Health,** Oxford, v. 2, n. 2, p. 1699-1707, 2006. https://doi.org/10.1111/j.1365-3156.2006.01727.x

FORDE, B. M.; O'TOOLE, P. W. Next-generation sequencing technologies and their impact on microbial genomics. **Briefings in functional genomics**, v. 12, n. 5, p. 440-453, 2013. Disponível em: https://doi.org/10.1093/bfgp/els062

FOUTS, D.E., MATTHIAS, M.A., ADHIKARLA, H., ADLER, B., AMORIM-SANTOS, L., BERG, D.E., et al. What Makes a Bacterial Species Pathogenic?:Comparative Genomic Analysis of the Genus Leptospira. **PLoS Negl Trop Dis**. v. 10, n. 2:

e0004403, 2016. https://doi.org/10.1371/journal.pntd.0004403

FRAGA, T. R.; BARBOSA, A. S.; ISAAC, L. Leptospirosis: Aspects of innate immunity, immunopathogenesis and immune evasion from the complement system. **Scand J Immunol**, v.73, n.5, p.408-419, 2011. Disponível em: https://doi.org/10.1111/j.1365-3083.2010.02505.x

GAMBERINI, M.; GOMEZ, R.M.; ATZINGEN, M.V.; MARTINS, E.A.L; VASCONCELLOS, S.A.; ROMERO, E.C.; LEITE, L.C.C.; HO, P.L.; NASCIMENTO, A.L.T.O. Whole-Genome analysis of Leptospira interrogans to identify potential vaccine candidates against leptospirosis. **FEMS Microbiol. Lett.,** v. 244, n. 2, p. 305-13, 2005. Disponível em: https://doi.org/10.1038/srep20020

GONZÁLEZ, A., BORRERO, R., RUIZ, J., BATISTA, N., FERNÁNDEZ, Y., VALDÉS, Y., et al. Medio EMJH modificado para el cultivo de Leptospira interrogans serogrupo Ballum. **Rev. Argent**. **Microbiol**. v. 38, p. 61–68, 2006.

GUIDUGLI, F. **Prevenção e tratamento da leptospirosis: revisão sistemática de ensaios clínicos aleatórios com metanálises.** São Paulo, SP. Tese Doutorado - Universidade Federal de São Paulo/Escola Paulista de Medicina, 2000.

GRANT, J. R., ARANTES, A. S., & STOTHARD, P. Comparing thousands of circular genomes using the CGView Comparison Tool. **BMC genomics**, v. 13, n. 1, p. 202, 2012. Disponível em: https://doi.org/10.1186/1471- 2164-13-202

GRASSMANN, A.A., FELIX, S.R., DOS SANTOS, C.X., AMARAL, M.G., et al. Protection against lethal leptospirosis after vaccination with LipL32 coupled or coadministered with the B subunit of *Escherichia coli* heat-labile enterotoxin. **Clin Vaccine Immunol**, v. 19, n. 5, p. 740–745, 2012. Disponível em: https://doi.org/10.1128/CVI.05720-1

GRASSMANN, A.A., SOUZA, J.D., MCBRIDE, A.J. A universal vaccine against leptospirosis: are we going in the right direction? **Front Immunol**, v. 8, p. 256, 2017b. Disponível em: https://doi.org/10.3389

GRASSMANN, A.A., KREMER, F.S., DOS SANTOS, J.C., SOUZA, J.D., PINTO, L.D.S., MCBRIDE, A.J. Discovery of Novel Leptospirosis Vaccine Candidates Using Reverse and Structural Vaccinology. **Front Immunol**. v. 8, p. 463 2017a. Disponível em: https://doi.org/10.3389/fimmu.2017.00463.

GREENE, C.E. Leptospirosis. **In: Infections Diseases of the dog and cat**, 4 ed. Saunders Elsevier: St Louis, p.402-417, 2011.

HAAKE, D.A., MAZEL, M.K., MCCOY, A.M., et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. **Infection and immunity**, v. 67, n. 12, p. 6572–82, 1999.

HAAKE, D.A. Spirochaetal lipoproteins and pathogenesis. **Microbiology**, v. 146, p. 1491-1504, 2000. https://doi.org/10.1099/00221287-146-7-1491

HAAKE, D. A.; MATSUNAGA, J. Leptospira: A spirochaete with a hybrid outer membrane. **Mol Microbiol**, v. 77, n. 4, p. 805-814, 2010.

https://doi.org/10.1111/j.1365-2958.2010.07262.x

HAMOND, C., PESTANA, C.P., MEDEIROS, M.A., LILENBAUM, W. Genotyping of Leptospira directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. **Epidemiol. Infect**. v. 16, p. 1–4, 2015. https://doi.org/10.1017/S0950268815001363

HARTWIG, D.D., SEIXAS, F.K., CERQUEIRA, G.M., MCBRIDE, A.J., DELLAGOSTIN, O.A. Characterization of the immunogenic and antigenic potential of putative lipoproteins from Leptospira interrogans. **Curr. Microbiol**. v. 62, p. 1337–1341, 2011. Disponível em: https://doi.org/10.1007/s00284-010-9865-1

HARTWIG, D.D., FORSTER, K.M., OLIVEIRA, T.L., AMARAL, M., MCBRIDE, A.J., DELLAGOSTIN, O.A. A prime-boost strategy using the novel vaccine candidate, lema, protects hamsters against leptospirosis. **Clin. Vaccine Immunol**. v. 20, p. 747–752, 2013. Disponível em: https://doi.org/10.1128/CVI.00034-13.

HASHIMOTO, V.L. **Clonagem e expressão gênica de antígenos candidatos vacinais contra leptospirose.** [Tese (Doutorado em Biotecnologia)] – São Paulo – Instituto de Ciências Biomédicas da Universidade de Paulo, 2012.

HASHIMOTO V.Y., CHIDEROLI, R.T., RIBEIRO, J., ALFIERI A.A., COSTA G.M., FREITAS, J.C., et al. Serological and molecular findings in diagnosis of leptospirosis serovar hardjo in a dairy bovine herd. **Semin. Cien. Agrar**. v. 38, p. 3155–3164, 2017. Disponível em: https://doi.org/10.5433/1679-0359.2017v38n5p3155

HERRMANN, J.L., BELLENGER, E., PEROLAT, P., BARANTON, G., SAINT, G.I. Pulsed-field gel electrophoresis of NotI digests of leptospiral DNA: a new rapid method of serovar identification. **J. Clin. Microbiol**. v. 30, p. 1696–1702, 1992.

ILS- International leptospirosis society : https://www.leptosociety.org/resources, link: Minutes of TSC meeting, November 2017 (pdf)

JENSEN, L. J., FRIIS, C.; USSERY, D. W. Three views of microbial genomes. **Research in Microbiology**, v. 150, p. 773–777, 1999. https://doi.org/10.1016/s0923-2508(99)00116-3

JOHNSON, R.C., ROGERS, P. 5-Fluorouracil as a selective agent for growth of Leptospirae. **J. Bacteriol**. v. 87, p. 422–426, 1964.

JOHNSON, R.C., WALBY, J., HENRY, R.A., AURAN, N.E. Cultivation of parasitic leptospires: effect of pyruvate. **Appl. Microbiol**. v.26, p. 118–119, 1973.

JORGENSEN, W.L. The many roles of computation in drug discovery. **Science**, v. 303, n. 5665, p. 1813–1818, 2004. https://doi.org/10.1126/science.1096361

KITCHEN, D.B., DECORNEZ, H., FURR, J.R., BAJORATH, J. Docking and scoring in virtual screening for drug discovery: methods and applications. **Nat Rev Drug Discov**, v. 3, n. 11, p. 935–949, 2004. https://doi.org/10.1038/nrd1549

KO, A.I.; GOARANT, C.; PICARDEAU, M. Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. **Nature Reviews Microbiology**, v.

7, n. 10, p. 736-747, 2009. Disponível em: https://doi.org/10.1038/nrmicro2208

KOIZUMI, N., WATANABE, H. Leptospiral immunoglobulin-like proteins elicit protective immunity. **Vaccine**, v. 22, p. 1545–52, 2004. Disponível em: https://doi.org/10.1016/j.vaccine.2003.10.007

KOONIN, E. V.; WOLF, Y. I. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. **Nucleic acids research**, v. 36, n. 21, 6688–719, 2008. Disponível em: https://doi.org/10.1093/nar/gkn668

KOSITANONT, U. et al. Detection and differentiation between pathogenic and saprophytic Leptospira spp. by multiplex polymerase chain reaction. **Diagn. Microbiol. Infect. Dis**., v. 57, n. 2, p. 117-122, 2007. Disponível em: https://doi.org/10.1016/j.diagmicrobio.2006.07.014

KRAEMER, S.A., RAMACHANDRAN, A., PERRON, G.G. Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. **Microorganisms**. v. 7, n. 6, p. 180, 2019. Disponível em: https://doi.org/10.3390/microorganisms7060180

KUMAR, K.V., LALL, C., RAJ, R.V., VEDHAGIRI, K., SUNISH, I.P., VIJAYACHARI, P. In Vitro Antimicrobial Susceptibility of Pathogenic Leptospira Biofilm. Microbial Drug Resistance, v. 22, n. 7, p. 511-514, 2016. Disponível em: https://doi-org.ez78.periodicos.capes.gov.br/10.1089/mdr.2015.0284

LAMBERT, A.; PICARDEAU, M.; HAAKE, D. A.; SERMSWAN, R. W.; SRIKRAM, A.; ADLER, B.; MURRAY, G. A. Flaa proteins in leptospira interrogans are essential for motility and virulence but are not required for formation of the flagellum sheath. In**fect Immun**, v. 80, n. 6, p. 2019-2025, 2012. Disponível em: https://doi.org/10.1128/IAI.00131-12

LIAO, S.; SUN, A.; OJCIUS, D. M.; WU, S.; ZHAO, J.; YAN, J. Inactivation of the fliy gene encoding a flagellar motor switch protein attenuates mobility and virulence of leptospira interrogans strain lai. **BMC Microbiol**, v. 9, p. 253, 2009. Disponível em: https://doi.org/10.1186/1471-2180-9-253

LIEGEON, G., DELORY, T., PICARDEAU, M. Antibiotic susceptibilities of livestock isolates of leptospira. **International Journal of Antimicrobial Agents**, v. 51, p. 693-699, 2018. Disponível em: https://doi.org/10.1016/j.ijantimicag.2017.12.024

LEONARD, F.C., QUINN, P.J., ELLIS, W.A., O'FARRELL, K. Duration of urinary excretion of leptospires by cattle naturally or experimentally infected with *Leptospira interrogans* serovar hardjo. **Vet. Rec**. v. 131, p. 435–439, 1992. Disponível em: https://doi.org/10.1136/vr.131.19.435

LEVETT, P.N. Leptospirosis. **Clin. Microbiol. Rev**., v. 14, p. 296-326, 2001.

LEVETT, P.N. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. **Clin. Infect. Dis**., v. 36, p. 447-452, 2003. Disponível em: https://doi.org/10.1086/346208

LILENBAUM, W. Bovine Leptospirosis in Brazil: A Review, **Brazilian Journal of Veterinary Medicine**, v.18, n.1, p.9-13, 1996.

LÓPEZ D, VLAMAKIS H, KOLTER R. Biofilms. Cold Spring Harb Perspect Biol. 2010;2(7):a000398. doi:10.1101/cshperspect.a000398

MACIEL, E.A.P., DE CARVALHO, A.L.F., NASCIMENTO, S.F., DE MATOS, R.B., GOUVEIA, E.L., REIS, M.G., KO, A.I: Household transmission of leptospira infection in urban slum communities. **PLoS Negl Trop Dis**, v. 2: e154-https://doi.org/10.1371/journal.pntd.0000154, 2008.

MAJED, Z., BELLENGER, E., POSTIC, D., POURCEL, C., BARANTON, G., PICARDEAU, M. Identification of variable-number tandem-repeat loci in Leptospira interrogans sensu stricto. **J. Clin. Microbiol**. 43, 539–545, 2005. Disponível em: https://doi.org/10.1128/JCM.43.2.539-545.

MARTINS, G., LILENBAUM, W. The panorama of animal leptospirosis in Rio de Janeiro, Brazil, regarding the seroepidemiology of the infection in tropical regions. **BMC Vet Res,** v. 9, p. 237, 2013. Disponível em: https://doi.org/doi:10.1186/1746-6148-9-237

MARTINEZ-LOPEZ, D. G.; FAHEY, M.; COBURN, J. Responses of human endothelial cells to pathogenic and non-pathogenic Leptospira species. **PLoS Neglected Tropical Diseases**, v. 4, n. 12, p. e918, 2010.

MATHEWS, K.A.; MONTEITH, G. Evaluation of adding diltiazem therapy to standard treatment of acute renal failure caused by leptospirosis: 18 dogs (1998-2001). **Journal of Veterinary Emergency and Critical Care**, v.17, n.2, p.149-158, 2007. https://doi.org/10.1111/j.1476-4431.2007.00232.x

MCCONKEY, B.J., SOBOLEV, V., EDELMAN, M. The performance of current methods in ligand-protein docking. **Current Science**, v. 83, p. 845–855, 2002.

MEDINI, D., DONATI, C., TETTELIN, H., MASIGNANI, V., RAPPUOLI, R. The microbial pan-genome. **Current Opinion in Genetics & Development**. v.15, n. 5, p. 589-594, 2005. Disponível em: https://doi.org/10.1016/j.gde.2005.09.006.

MENG, X.Y.; ZHANG, H.X.; MEZEI, M.; CUI, M. Molecular docking: A powerful approach for structure-based drug discovery. **Curr. Comput. Aided Drug Des**, v. 7, p. 146–157, 2011. Disponível em: https://doi.org/10.2174/157340911795677602

MÉRIEN, F.; AMOURIAUX, P.; PEROLAT, P.; BARANTON, G.; SAINTGIRONS, I. Polymerase chain reaction for detection of Leptospira spp. in clinical samples. **J. Clin. Microbiol**., v. 30, n. 9, p. 2219-2224, 1992.

METZKER, M. L. Emerging technologies in DNA sequencing. **Genome Research**, v. 15, n. 12, p. 1767–1776, 2005. https://doi.org/10.1101/gr.3770505

MIRAGLIA, F., DE MORAES, Z.M., MELVILLE, P. A., DIAS, R.A., VASCONCELLOS, S.A. Emjh medium with 5-fluorouracil and nalidixic acid associated with serial dilution technique used to recover *leptospira* spp from experimentally contaminated bovine semen. **Braz. J. Microbiol**. v. 40, p. 189–193, 2009. Disponível em: https://doi.org/10.1590/S1517-83822009000100033

MOITESSIER, N., ENGLEBIENNE, P., LEE, D., LAWANDI, J., CORBEIL, C.R.

Towards the development of universal, fast and highly accurate docking/scoring methods: a long way to go. **Br J Pharmacol**, v. 153, n. 1, p. 7–26, 2008. Disponível em: https://doi.org/10.1038/sj.bjp.0707515

MUNTHA P. Drug discovery & development. **Journal of Pharmacy and Pharmaceutical Sciences**. v. 5, n. 1, p. 135-142, 2016.

MURRAY, C.K., HOSPENTHAL, D.R. Broth microdilution susceptibility testing for Leptospira spp. **Antimicrob Agents Chemother** v. 48, p. 1548–1552, 2004. Disponível em: 10.1128/AAC.48.5.1548-1552.2004

MURRAY, G.L., LO, M., BULACH, D.M., SRIKRAM, A., SEEMANN, T., et al. Evaluation of 238 antigens of Leptospira borgpetersenii serovar Hardjo for protection against kidney colonisation. **Vaccine**. v. 31, p. 495–499, 2013. Disponível em: https://doi.org/10.1016/j.vaccine.2012.11.028.

MYERS D. M. Efficacy of combined furazolidone and neomycin in the control of contamination in Leptospira cultures. **Antimicrob. Agents Chemother**. v.7, p. 660–671, 1975. Disponível em: https://doi.org/10.1128/AAC.7.5.666

NASCIMENTO, A.L., KO, A.I., MARTINS, E.A., MONTEIRO-VITORELLO, C.B., HO, P.L., HAAKE, D.A., et al. Comparative genomics of two Leptospira interrogans serovars reveals novel insights into physiology and pathogenesis. **J Bacteriol**. v. 186, n. 7, p. 2164-72, 2004a. https://doi.org/10.1128/jb.186.7.2164-2172.2004

NASCIMENTO, A.L.T.O.; VERJOVSKI-ALMEIDA, S.; VAN SLUYS, M.A.; MONTEIRO-VITORELLO, C.B.; CAMARGO, L.E.A.; DIGIAMPIETRI, L.A.; HARSTKEERL, R.A.; HO, P.L.; MARQUES, M.V.; OLIVEIRA, M.C.; SETUBAL, J.C.; HAAKE, D.A.; MARTINS, E.A.L. Genome features of Leptospira interrogans serovar Copenhageni. **Braz. J. Med. Biol. Res**., v. 37, n. 4, p. 459-477, 2004b. http://dx.doi.org/10.1590/S0100-879X2004000400003

NISHIDA, H. Genome DNA Sequence Variation, Evolution, and Function in Bacteria and Archaea. **Current issues in molecular biology**, v. 15, n. 1, p. 19–24, 2012.

GONÇALVES, D. D. et al. Soroepidemiologia e variáveis ocupacionais e ambientais relacionadas à leptospirose, brucelose e toxoplasmose em trabalhadores de frigorífico do Estado do Paraná, Brasil. Revista do Instituto de Medicina Tropical de São Paulo, v. 48, n. 3, p. 135-140, 2006.

OLIVEIRA, J.F.P; CIPULLO, J.P; BURDMANN, E.A. Nefrotoxicidade dos aminoglicosídeos. **Braz J Cardiovasc Surg,** v. 21, n. 4, p. 444-452, 2006.

OLIVEIRA, S.T. **Leptospirose canina: dados clínicos laboratoriais e terapêuticos em cães naturalmente infectados.** 2010. 88 folhas. Tese (doutorado) – Universidade Federal do Rio Grande do Sul, Faculdade de Veterinária, Programa de Pós-graduação em Ciências Veterinárias, Porto Alegre, 2010.

OLIVEIRA, T.L., GRASSMANN, A.A., SCHUCH, R.A., SEIXAS NETO, A.C., MENDONCA, M., HARTWIG, D.D., et al. Evaluation of the Leptospira interrogans outer membrane protein OmpL37 as a vaccine candidate. **PLoS One** v. 10, n. 11,p. e0142821, 2015. Disponível em: https://doi.org/10.1371/journal.pone.0142821

OOTEMAN, M.C.; VAGO, A.R.; KOURY, M.C. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. **Journal of Microbiological Methods**, Amsterdam, v. 65, n. 2, p. 247-257, 2006. Disponível em: https://10.1016/j.mimet.2005.07.015

ORDÓÑEZ NG. Immunohistochemical endothelial markers: a review. **Adv Anat Pathol**. 2012;19:281-95.

PAES, A.C. Leptospirose canina. *In:* MEGID, J; RIBEIRO, M.G; PAES, A.C. **Doenças Infecciosas em Animais de Produção e de Companhia**. Rio de Janeiro: Editora Roca, 1ed, p. 356-377, 2016.

PAGANI, I., LIOLIOS, K., JANSSON, J., CHEN, I.-M. A., SMIRNOVA, T., NOSRAT, B., MARKOWITZ, V. M., et al. (2012). The Genomes OnLine Database (GOLD) v.4: status of genomic and metagenomic projects and their associated metadata. **Nucleic acids research, (Database issue),** D571–9. https://doi.org/10.1093/nar/gkr1100.

PAILHORIÈS H., BUZELÉ R., PICARDEAU M., ROBERT S., MERCIER E., MEREGHETTI L., et al. Molecular characterization of *Leptospira* sp by multilocus variable number tandem repeat analysis (MLVA) from clinical samples: a case report. **Int. J. Infect. Dis**. v. 37, p. 119–121, 2015. Disponível em: https://doi.org/10.1016/j.ijid.2015.06.026

PALLEN, M. J.; WREN, B. W. Bacterial pathogenomics. **Nature**, v. 449, p. 835–842, 2007. Disponível em: https://doi.org/10.1038/nature06248

PARTE, A.C. LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. **International Journal of Systematic and Evolutionary Microbiology**, v. 68, p. 1825-1829, 2018. Disponível em: doi: 10.1099/ijsem.0.002786

PEROLAT, P., LECUYER, I., POSTIC, D., BARANTON, G. Diversity of ribosomal DNA fingerprints of Leptospira serovars provides a database for subtyping and species assignation. **Res. Microbiol**. v. 144, p. 5–15, 1993. Disponível em: https://doi.org/10.1016/0923-2508(93)90210-S

PICARDEAU, M., BULACH, D.M., BOUCHIER, C., ZUERNER, R.L., ZIDANE, N., et al. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. **PLoS One**. v. 3, n. 2, p. e1607, 2008. Disponível em: https://doi.org/10.1371/

PICARDEAU, M. Diagnosis and epidemiology of leptospirosis. **Med. Mal. Infect**. v. 43, p. 1–9, 2013. Disponível em: https://doi.org/ 10.1016/j.medmal.2012.11.005

PLANK, R.; DEAN, D. Overview of the epidemiology, microbiology, and pathogenesis of Leptospira spp. in humans. **Microbes and Infection**, v. 2, p.1265-1276, 2000. Disponível em: https://doi.org/10.1016/s1286-4579(00)01280-6

POSTIC, D., RIQUELME-SERTOUR, N., MERIEN, F., PEROLAT, P., BARANTON, G. Interest of partial 16S rDNA gene sequences to resolve heterogeneities between Leptospira collections: application to L. meyeri. **Research in Microbiology**, v. 151, p. 333-341, 2000. Disponível em: https://doi.org/10.1016/s0923-2508(00)00156-x

PUCHE, R., FERRÈS, I., CARABALLO, L., RANGEL, Y., PICARDEAU, M., et al. Leptospira venezuelensis sp. nov., a new member of the intermediates group isolated from rodents, cattle and humans. **International Journal Of Systematic And Evolutionary Microbiology,** v. 68, n. 2, 2018. Disponível em: https://doi.org/doi:10.1099/ijsem.0.002528

RAJA, V., NATARAJASEENIVASAN. K. Pathogenic, diagnostic and vaccine potential of leptospiral outer membrane proteins (OMPs) **Crit. Rev. Microbiol**. v. 41, p. 1–17, 2015. Disponível em: https://doi.org/10.3109/1040841X.2013.787387.

RALPH, D., MCCLELLAND, M., WELSH, J., BARANTON, G., PEROLAT, P. Leptospira species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. **J. Bacteriol**. v. 175, p. 973–981, 1993. Disponível em: https://doi.org/10.1128/jb.175.4.973-981.

RAPPUOLI, R. Reverse vaccinology, a genome-based approach to vaccine development. **Vaccine**, v.19, p. 2688–91, 2001. Disponível em: https://doi.org/10.1016/S0264-410X(00)00554-5

RATET, G., VEYRIER, F.J., FANTON D'ANDON, M., KAMMERSCHEIT, X., NICOLA, M.A., PICARDEAU, M., et al. Live imaging of bioluminescent leptospira interrogans in mice reveals renal colonization as a stealth escape from the blood defenses and antibiotics. **PLoS Negl Trop Dis**. v. 8, n. 12, p. e3359, 2014. Disponível em: https://doi.org/10.1371/journal.pntd.0003359

REN, S.X., FU, G., JIANG, X.G., ZENG, R., MIAO, Y.G., et al. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. **Nature**. v. 422, p. 888–893, 2003. Disponível em: https://doi.org/10.1038/nature01597

REZASOLTANI, S., DABIRI, H., KHAKI, P., ROSTAMI NEJAD, M., KARIMNASAB, N., & MODIRROUSTA, S. Characterization of Leptospira interrogans Serovars by Polymorphism Variable Number Tandem Repeat Analysis. **Jundishapur journal of microbiology,** v. 8, n. 10, e22819, 2015. Disponível em: https://doi:10.5812/jjm.22819.

RIEDIGER, I.N. **Comparação dos diagnósticos sorológico e molecular da leptospirose humana na região metropolitana de curitiba, paraná.** Paraná, PR. Dissertação de Mestrado - Universidade Federal do Paraná, 2007.

RISTOW, P.; BOURHY, P.; MCBRIDE, F.W.; FIQUEIRA, C.P., HUERRE, M.; AVE, P. GIRONS, I.S.; KO, A.I.; PICARDEAU, M. The OmpA-like protein Loa22 is essential for leptospiral virulence. **PloS Pathog**., v. 3, n. 7, p. 97, 2007. Disponível em: https://doi.org/10.1371/journal.ppat.0030097

ROULI, L., MERHEJ, V., FOURNIER, P.E., RAOULT, D. The bacterial pangenome as a new tool for analysing pathogenic bacteria. **New Microbes New Infect**. v. 7, p. 72–85, 2015. Disponível em: https://doi.org/10.1016/j.nmni.2015.06.005

SEIXAS, F.K., FERNANDES, C.H., HARTWIG, D.D., CONCEICAO, F.R., ALEIXO, J.A., DELLAGOSTIN, O.A. Evaluation of different ways of presenting LipL32 to the

immune system with the aim of developing a recombinant vaccine against leptospirosis. **Can J Microbiol**, v. 53, n. 4, p. 472–9, 2007. Disponível em: https://doi.org/0.1139/w06-138

SINGH, N., WARSHEL, A. Absolute binding free energy calculations: on the accuracy of computational scoring of protein-ligand interactions. **Proteins**, v. 78, n. 7, p. 1705–1723, 2010. Disponível em: https://doi.org/10.1002/prot.22687

SOBOLEVA, G.L. Preparation and use of solid culture medium for *Leptospira* isolation. **Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii**, v. 2, p. 80-84, 1983.

SRITRAKULA, T., WAJJWALKUD, S.N.W., et al. *Leptospira borgpetersenii* hybrid leucine-rich repeat protein: Cloning and expression, immunogenic identification and molecular docking evaluation. **Journal of Microbiological Methods**, v. 142, p. 52–62, 2017. Disponível em: http://dx.doi.org/10.1016/j.mimet.2017.09.005

STANECK, J.L., HENNEBERRY, R.C., COX, C.D. Growth requirements of pathogenic leptospira. **Infect. Immun**. v. 7, p. 886–897, 1973.

SUEPAUL, S.M., CARRINGTON, C., CAMPBELL, M., BORDE, G., ADESIYUN, A.A. Antimicrobial susceptibility of leptospira isolates from dogs and rats to 12 antimicrobial agents. **Trop Biomed**, v. 32, p. 1–10, 2015.

TAVARES, W. **Manual de Antibióticos e Quimioterápicos Antiinfecciosos**.3a.ed São Paulo: Atheneu, 746p, 2014.

TETTELIN, H., MASIGNANI, V., CIESLEWICZ, M. J., DONATI, C., MEDINI, D., WARD, N. L., ANGIUOLI, S. V, et al. Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial “pan-genome”. **Proceedings of the National Academy of Sciences of the United States of America**, v. 102, n. 39, p. 13950–5, 2005. Disponível em: https://doi.org/10.1073/pnas.0506758102

THIBEAUX, R., IRAOLA, G., FERRÉS, I., BIERQUE, E., GIRAULT, D., SOUPÉ-GILBERT, M.E., et al. Deciphering the unexplored Leptospira diversity from soils uncovers genomic evolution to virulence. **Microb Genom**, v. 9, p. 816, 2018. Disponível em: https://doi.org/10.3389/fmicb.2018.00816

THIERMANN, A.B., HANDSAKER, A.L., MOSELEY, S.L., KINGSCOTE, B. New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar kennewicki. **J. Clin. Microbiol**. v. 21, p. 585–587, 1985.

TOUCHMAN, J. Comparative Genomics. **Nature Education Knowledge**, v. 3, n. 10, p. 13, 2010.

TURNER L. H. Leptospirosis III: Maintenance, isolation and demonstration of leptospires. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 64, p. 623-646, 1970. https://doi.org/10.1016/0035-9203(70)90087-8

TRONCOSO, A., CASTRELO, M.J. Leptospirosis: A re-emerging zoonosis. **Journal**

of Coastal Life Medicine, v. 4, n. 9, p. 673-677, 2016. Disponível em: https://doi.org/10.12980/jclm.4.2016J6-157

UKHOVSKYI, V., TSARENKO, T., VYDAYKO, N., KORNIIENKO, L., NEBOGATKIN, I. Genotyping of pathogenic leptospira by Multiple Locus Variable-number Tandem Repeat Analysis (MLVA). **Online J Public Health Inform**, v. 10, n. 1, p. e131, 2018. Disponível em: https://doi.org/10.5210/ojphi.v10i1.8902.

USSERY, D. W., WASSENAAR, T. M., BORINI, S. Microbial Genome Sequences: A New Era in Microbiology. *In:* USSERY, D. W., WASSENAAR, T. M., BORINI, S. **Computing for Comparative Microbial Genomics: Bioinformatics for Microbiologists**. Springer London. 1 ed. p. 37-51, 2009.

VANAPORN W., PREMJIT A., DANIEL H.P., SAYAN L., JANJIRA T., WIRONGRONG C., LEE D.S., NICHOLAS J.W., NICHOLAS P.D., AND DIREK L., et al. Rapid isolation and susceptibility testing of Leptospira spp. using a new solid medium, LVW agar. **Antimicrob. Agents Chemother**. v. 57, p. 297–302, 2013. Disponível em: https://doi.org/ 10.1128/AAC.01812-12

VICTORIA, B., AHMED, A., ZUERNER, R.L., AHMED, N., BULACH, D.M., QUINTEIRO, J., HARTSKEERL, R.A. Conservation of the S10-spc-alpha locus within otherwise highly plastic genomes provides phylogenetic insight into the genus Leptospira. **PLoS One**, v. 3, e2752, 2008. Disponível em: https://doi.org/10.1371/journal.pone.0002752.

VINCENT, A.T.; SCHIETTEKATTE, O.; GOARANT, C.; NEELA, V.K.; BERNET, E.; THIBEAUX, R.; ISMAIL, N.; MOHD KHALID, M.K.N.; AMRAN, F.; MASUZAWA, T.; et al. Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. **PLoS Negl. Trop. Dis***.* v.13, p. e0007270, 2019. https://doi.org/10.1371/journal.pntd.0007270

VIEIRA, M.L. **Análise da expressão de proteínas de Leptospira interrogans virulentas e avirulentas pela proteômica.** 2008. Dissertação (Mestrado em Biotecnologia) – Centro de Biotecnologia, Instituto Butantan, São Paulo, Brasil, 2008.

XU, Y., ZHU, Y., WANG, Y., et al. Whole genome sequencing revealed host adaptation-focused genomic plasticity of pathogenic Leptospira. **Sci Rep.** v.6, p.200-20, 2016. Disponível em: https://doi.org/10.1038/srep20020.

WORLD HEALTH ORGANIZATION – ILS. WHO. Human leptospirosis: Guidancefor 963 diagnosis, surveillance and control. **World Health Organization**, 2003.

WOHL, J.S. Canine Leptospirosis. *IN:* **Revista Compendium**. Vol. 18. nº. 11. p. 1215-225, Novembro, 1996.

YAN J, DAI B, YU E. Leptospirosis. 3rd Ed Beijing: People's Medical Publisheing House; 2006.

YANG, H.L.; ZHU, Y.Z.; QIN, J.H.; JAING, X.C.; ZHAO, G.P.; GUO, X.K. In silico and microarray-based genomic approaches to identifying potential vaccine candidates against Leptospira interrogans. **BMC Genomics**, v. 7, p. 293-305, 2006. Disponível em: https://doi.org/ 10.1186/1471-2164-7-293

ZACARIAS, F.G.D.S., VASCONCELLOS, S.A., ANZAI, E.K., GIRALDI, N., DE FREITAS, J.C., HARTSKEERL, R. Isolation of Leptospira serovars Canicola and Copenhageni from cattle urine in the State of Parana, Brazil. **Braz. J. Microbiol**. v. 39, p. 744–748, 2008. Disponível em: https://doi.org/10.1590/S1517-83822008000400028

ZARANTONELLI, L., SUANES, A., MENY, P., BURONI, F., NIEVES, C., SALABERRY, X., et al. Isolation of pathogenic Leptospira strains from naturally infected cattle in Uruguay reveals high serovar diversity, and uncovers a relevant risk for human leptospirosis. **PLoS Negl Trop Dis,** v. 12, n. 9, p. e0006694, 2018. Disponível em: https://doi.org/10.1371/journal.pntd.0006694

ZHANG C, WANG H, YAN J. Leptospirosis prevalence in Chinese populations in the last two decades. Microbes Infect. 14:317–23, 2012. doi:10.1016/j.micinf.2011.11.007

3 HIPÓTESES

- O uso de suplementos (piruvato de sódio, soro fetal bovino e enzima superóxido dismutase) adicionados ao meio de cultura padrão de isolamento de leptospiras é útil no crescimento inicial e subsequente manutenção de sorovares mais fastidiosos, como por exemplo o sorovar Hardjo.

- A adição de suplementos (piruvato de sódio, soro fetal bovino e enzima superóxido dismutase) ao meio de cultura padrão de isolamento de leptospiras pode influenciar na sua viabilidade, motilidade e morfologia

- A técnica de MLVA (*Multilocus variable-number tandem-repeat analysis*) associada ao sequenciamento do gene *secY* é eficiente para caracterização molecular e tipificação de genotipos de sorovares pertencentes a espécies diferentes de *Leptospira.*

- O uso de ferramentas de bioinformática é uma boa alternativa para otimizar a predição de alvos vacinais e drogáveis em microrganismos que tem um crescimento lento *in vitro,* assim como o gênero *Leptospira*.

- A abordagem com metodologias *in silico* pode agilizar a busca por novas drogas que ajudem tanto na recuperação clínica quanto na eliminação da leptospiras dos túbulos renais.

5 OBJETIVOS

5.1 OBJETIVO GERAL

- Desenvolver uma nova estratégia de cultura e isolamento de sorovares de leptospira mais fastidiosos a fim de possibilitar a caracterização molecular, sequenciamento gênomico e busca de alvos vacinais e drogáveis *in silico*.

5.2 OBJETIVOS ESPECÍFICOS

- Aumentar as taxas de isolamento inicial e manutenção de sorovares mais fastidiosos adicionando suplementos (piruvato de sódio, soro fetal bovino e enzima superóxido dismutase) ao meio de cultura EMJH;

- Verificar os efeitos dos suplementos adicionais na morfologia, viabilidade e motilidade das leptospiras;

- Utilizar técnicas de biologia molecular, como MLVA e sequenciamento do gene *secY,* para caracterização e tipificação de isolados do sorovar Hardjo;

- Sequenciar o genoma de duas leptospiras, *L. interrogans* sorovar Hardjo genotipo Hardjoprajitno e *L. borgpetersenii* sorovar Hardjo genotipo Hardjobovis através da tecnologia de sequenciamento de nova geração;

- Comparar os dois genomas sequenciados do sorovar Hardjo com outros genomas de *Leptospira* depositados no banco de dados *GenBank* utilizando a abordagem da gênomica comparativa.

- Utilizar ferramentas de bioinformática e abordagens de genômica subtrativa para descobrir novos candidatos vacinais e alvos para novas drogas com intuito de auxiliar no controle e tratamento da leptospirose animal.

6 ARTIGO A – CULTURE STRATEGIES FOR ISOLATION OF FASTIDIOUS LEPTOSPIRA SEROVAR HARDJO AND MOLECULAR DIFFERENTIATION OF GENOTYPES HARDJOBOVIS AND HARDJOPRAJITNO

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Culture Strategies for Isolation of Fastidious *Leptospira* Serovar Hardjo and Molecular Differentiation of Genotypes Hardjobovis and Hardjoprajitno

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The Leptospira serovar Hardjo belongs to the serogroup sejroe and this serovar is the most prevalent in bovine herds worldwide. The sejroe serogroup is the most frequently detected by serology in Brazilian cattle herds suggesting that it is due serovar Hardjo. In the molecular classification, this serovar has two genotypes: Hardjobovis and Hardjoprajitno. This serovar is as considered as fastidious pathogens, and their isolation is one of the bottlenecks in leptospirosis laboratories. In addition, its molecular characterization using genomic approaches is oftentimes not simple and time-consuming. This study describes a method for isolating the two genotypes of serovar Hardjo using culture medium formulations and suggests a get-at-able molecular characterization. Ten cows naturally infected which were seropositive were selected from small dairy farms, and their urine was collected for bacterial isolation. We evaluated three modifications of liquid Leptospira medium culture supplemented with sodium pyruvate, superoxide dismutase enzyme and fetal bovine serum, and the isolates were characterized by molecular techniques. After isolation and adaptation in standard culture medium, the strains were subcultured for 1 week in the three modified culture media for morphologic evaluation using electronic microscopy. Strains were molecularly identified by multilocus variable-number tandem-repeat analysis (MLVA), partial sequencing and phylogenic analyses of gene sec Y. Combining the liquid culture medium formulations allowed growth of the Leptospira serovar Hardjo in three tubes. Two isolates were identified as genotype Hardjobovis, and the other as genotype Hardjoprajitno. Morphologically, compared with control media, cells in the medium supplemented with the superoxide dismutase enzyme were more elongated and showed many cells in division. The cells in the medium supplemented with fetal bovine serum were fewer and lost their spirochete morphology. This indicated that the additional supplementation with fetal bovine serum assisted in the initial growth and maintenance of the viable leptospires and the superoxide dismutase enzyme allowed

them to adapt to the medium. These culture strategies allowed for the isolation and convenient molecular characterization of two genotypes of serovar Hardjo, creating new insight into the seroepidemiology of leptospirosis and its specific genotypes. It also provides new information for the immunoprophylaxis of bovine leptospirosis.

Keywords: leptospirosis, culture medium, pyruvate sodium, superoxide dismutase, fetal bovine serum, DNA fingerprint

INTRODUCTION

The genus *Leptospira* comprises a heterogeneous group of pathogenic and saprophytic species belonging to the order *Spirochaetales* (Adler and de la Peña Moctezuma, 2010). Leptospiral serovar diversity results from structural heterogeneity in the carbohydrate component of the lipopolysaccharides (de la Peña-Moctezuma et al., 1999). Many serovars are adapted for specific mammalian hosts, which harbor these microorganisms in the renal tubules and intermittently eliminate them through the urine contaminating the surrounding environment (Adler and de la Peña Moctezuma, 2010).

Serovar Hardjo is one of serovars of sejroe serogroup. In bovine herds naturally infected, the serovar Hardjo is the most prevalent (Ellis, 2015). In Brazilian cattle herds, antibodies against the sejroe serogroup are the most frequently detected by the microscopic agglutination test (MAT) (Favero et al., 2001; Figueiredo et al., 2009; Hashimoto et al., 2012; Silva et al., 2012), suggesting that it is due to the serovar Hardjo. By molecular classification, this serovar has two genotypes (Hardjobovis and Hardjoprajitno). The genotype Hardjobovis belongs to the species *Leptospira borgpetersenii* and genotype Hardjoprajitno to the species *Leptospira interrogans*.

Serological methods are limited in that they can only distinguish the serovars at the serogroup level but cannot differentiate the genotypes of the Hardjo serovar (Picardeau, 2013), which are relevant for the epidemiology of these genotypes. Serovar determination is a very laborious methodology and the use of monoclonal antibody panels for the cross-agglutinin absorption test (CAAT), has a high cost for the implementation of this methodology and may only be performed at the Royal Tropical Institute reference laboratory in the Netherlands (Faine et al., 1999). Isolating leptospiral strains is useful for molecular characterization and genotyping; however, it is time-consuming and uncertain, particularly for the more fastidious serovars such as Hardjo (Pailhoriès et al., 2015). These microorganisms are slow-growing and require a rich medium at a neutral pH, which makes it difficult to cultivate leptospires from natural sources (Johnson and Gary, 1962; Staneck et al., 1973; Bey and Johnson, 1978; Adler et al., 1986; González et al., 2006; Zacarias et al., 2008).

Serovar Hardjo is difficult to culture and with low rates of success in the attempts of isolation due to its extreme nutritional requirements (Robertson et al., 1964; Flint and Liardet, 1980; Ellis and Thiermann, 1986; Leonard et al., 1992). While ordinary culture media are adequate to recover the less fastidious leptospires, they are ineffective for isolating serovar Hardjo.

Many studies are working to improve the culture media used for isolating *Leptospira* spp. by adding components that help the bacteria grow such as sodium pyruvate, different concentrations of Tween 80, bovine serum albumin (Johnson et al., 1973; Rodríguez et al., 2002; González et al., 2006; Wuthiekanun et al., 2014), and different combinations of antibiotics that inhibit contaminants (Johnson and Rogers, 1964; Myers, 1975; Zacarias et al., 2008; Miraglia et al., 2009; Chakraborty et al., 2011). However, there are few studies regarding the effect of these supplements on the initial isolation and maintenance of the strains as well as the supplement's influence on the viability, motility, and leptospiral cell morphology.

In the last three decades, molecular methods such as pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), Multiple Locus Sequence Typing (MLST), and multilocus variable-number tandem-repeat analysis (MLVA) were introduced to diagnosis, identification and characterization of leptospires (Thiermann et al., 1985; Herrmann et al., 1992; Perolat et al., 1993; Ralph et al., 1993; Majed et al., 2005; Ahmed et al., 2006). In particular, the MLVA method is useful, low time-consuming and has accessible costs for detecting and identifying *Leptospira* serovars (Pourcel et al., 2003), including the genotype Hardjobovis of serovar Hardjo (Chideroli et al., 2016).

This work describes different culture media compositions for isolating two genotypes of serovar Hardjo, their molecular characterization by MLVA, and their phylogeny using a partial sequence of the *secY* gene.

MATERIALS AND METHODS

Selection of Animals

Cows naturally infected from three small dairy farms in the northern Parana state were monitored serologically for leptospirosis due to their history of reproductive failure. Of these animals, 10 cows that tested positive on the MAT test (titers between 100 and 1,600 for serogroup sejroe) were selected, and their urine was collected for bacterial isolation and DNA detection by PCR.

Animal Ethics and Usage

The study was carried out in accordance with the recommendations of National Council for Control of Animal Experimentation (CONCEA). The protocol was approved by The Ethics Committee on Animal Use (CEUA) from State University of Londrina number CEEA - 58/06.

Preparation of the New Culture Medium

Three formulations of base liquid culture media were produced to isolate and maintain the leptospires. All media contained basic ingredients and additional supplements (**Table 1**).

Culture medium A contained *Leptospira* Medium Base culture Ellinghausen-McCullough-Johnson-Harris (EMJH) (2.56 g/L; Difco™, InterLab, BR), *Leptospira* Enrichment EMJH (100 mL/L; Difco™, InterLab, BR), and sodium pyruvate (0.1 g/L; Sigma ®, USA). Medium B contained the same components as medium A with the addition of the enzyme, superoxide dismutase (25,000 U/L; Sigma ®, USA). Medium C was similar to B; however, the base Enrichment EMJH (rabbit serum supplement) was changed to fetal bovine serum (100 mL/L; Gibco ®, USA).

When necessary, the following antibiotics were added to the three culture media formulations: 5-fluorouracil (400 mg/L, Sigma ®, USA), chloramphenicol (5 mg/L, Sigma ®, USA), nalidixic acid (50 mg/L, Sigma ®, USA), neomycin (10 mg/L, Sigma ®, USA), and vancomycin (10 mg/L, Acros ®, USA) (Zacarias et al., 2008).

Urine Collection and Culture

A urine sample from each animal was obtained by perineal massage and immediately seeded in tubes containing either culture medium A, B, and C with the five antibiotics. After incubation at 28°C for 24 h, the cultures were seeded in duplicate using the same three different culture media without antibiotics. The initial tubes with antibiotics were discarded after subculturing for 24 h, and the subcultures tubes were evaluated weekly for 6 months with a dark field microscope (Olympus BX40 Model).

Extraction and Amplification of DNA for MLVA and *secY*

For genetic characterization, DNA from the leptospire cultures was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen Life Technologies, Eugene, OR, USA). DNA from the leptospiral strain isolates was amplified using the Platinum PCR SuperMix Kit (Invitrogen Life Technologies, Eugene, OR, USA) according to the following conditions: 45 µL of each reaction containing SuperMix, 1 µL of each primer (10 nM), and 3 µL of DNA template (50 ng). All products were analyzed by electrophoresis in a 2% agarose gel with ethidium bromide (0.5 g/mL) in 0.5X TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA), pH 8.4, and visualized under ultraviolet light.

TABLE 1 | Resume of the combinations of the EMJH culture media.

Culture medium*	Formulation
A	EMJH base medium + base enrichment + sodium pyruvate
B	EMJH base medium + base enrichment + sodium pyruvate + superoxide dismutase enzyme
C	EMJH base medium + fetal bovine serum + sodium pyruvate + superoxide dismutase

*All culture media were produced both with and without antibiotics.

Molecular size was estimated by comparison with a 100-bp ladder.

Molecular Typing of the Isolates

To characterize the *Leptospira* strains, two molecular techniques were used. The MLVA identified isolates with five primer pairs for the VNTR loci 4, 7, 10, LB4, and LB5 as previously described (Salaün et al., 2006). For each of the five PCRs, the VNTR loci were used as positive controls for the reference strains of *L. interrogans* serovar Canicola serogroup canicola strain Canicola Hond Utrecht IV, *L. interrogans* serovar Hardjo serogroup sejroe genotype Hardjoprajitno strain Hardjoprajitno, and *L. borgpetersenii* serovar Hardjo serogroup sejroe genotype Hardjobovis strain Sponselee. After amplification, the sequencing of *secY* was used to identify and confirm genetic species, as previously described (Ahmed et al., 2006).

The products of the *secY* gene amplification were purified with a Purelink Genomic DNA extraction kit (Invitrogen Life Technologies, Eugene, OR, USA), quantified by a Qubit™ Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA), and sequenced on a ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using forward and reverse primers. Sequence quality was analyzed by the Phred program (http://asparagin.cenargen.embrapa.br/phph/). The consensus sequences were obtained by CAP3 software (http://asparagin. cenargen.embrapa.br/cgi-bin/phph/cap3.pl), and the identities were compared with the sequences in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identity matrix was created in the BioEdit program with the alignment and phylogenetic tree developed by the MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al., 2016).

Scanning Electron Microscopy (SEM)

After isolation, one isolate of each genotype (Hardjobovis and Hardjoprajitno) was subcultered in the three culture media formulations at 28°C for 7 days. Next, the cultures were centrifuged for 5 min at 2,000 rpm, resuspended in 100 µL of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.0) and transferred to 24-well polystyrene microtiter plates (Nunc, Roskilde, Denmark) with glass coverslips pre-coated with a thin layer of poly-L-lysine (Sigma Chemical Co, USA). After 1 h, the volume was adjusted to 500 µL of fixing solution to avoid cells adhering to the coverslips, and incubated at 25°C for 12 h. Samples were post-fixed in 1% OsO_4 (Electron Microscopy Sciences, Washington, PA, USA) and dehydrated in an ethanol series (30, 50, 70, 90, and 100°GL). Samples were critical-point dried with CO_2 (BALTEC CPD 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater), and observed under a SEM (FEI Quanta 200, Netherlands).

RESULTS

The combined formulations of EMJH liquid culture media allowed the growth of *Leptospira* serovar Hardjo strains from three farms (one strain of each farm). Posteriorly, these

three strains were characterized by molecular methods as *L. interrogans* genotype Hardjoprajitno (strain Londrina 53) and *L. borgpetersenii* genotype Hardjobovis (strains Londrina 49 and Londrina 54).

Table 2 shows that at the start of the experiment (12 ± 2 days), the first cells were unexpectedly seen in culture medium A with characteristics and movement similar to leptospires, while no cells were seen in culture media B or C. Thus, subculturing was performed using new tubes with the formulation media A, B, and C (A→A; A→B; A→C). After 21 ± 2 days, only subculture A→C presented leptospire cells, but they were few in number and with signs of suffering. On day 27 (±2), this tube was evaluated again and contained many leptospire cells; therefore, media A, B, and C were subcultered again (A→C→A; A→C→B; A→C→C). On day 34 (±3), there was no increase in cell number in subculture AC compared with subculture A→C→B, but there was an increased number of dead (unmoving) cells. In contrast, subculture A→C→B on day 34 (±3) presented many leptospiral cells and was transferred to new subcultures for medium B and C (A→C→B→B; A→C→B→C).

On day 41 (±2), the subculture A→C→B→C, unexpectedly presented more leptospiral cells per field than the subculture A→ C→ B→ B, and subculturing was performed on both tubes (A→C→B→C→B; A→C→B→C→C and A→C→B→B→B; A→C→B→B→C). On day 48 (±3), the subculture ACBC retained good cell growth as did subculture ACBCB. In contrast, subculture ACBCC had cells with less growth.

At the end of the experiment, all medium B subcultures, particularly A→C→B→C→B→B, presented excellent growth and gradually adapted to the standard routine media used in the laboratory without pyruvate sodium, superoxide dismutase, and fetal bovine serum. The isolated strains were named Londrina 49, Londrina 53, and Londrina 54.

Electron microscopy revealed that the leptospiral cells in culture medium B had a morphology that was more elongated as well as more cells, suggesting more bacterial cell division rate (**Figures 1A,C,E**). In contrast, there were fewer cells in medium C and those cells had lost the typical spirochete morphology (corkscrew-shaped with hooked ends) (**Figures 1B,D,F**). Morphologically, medium A was similar to B, but did not have a high number of cells and shown few elongated cells in division (Supplementary Figure 1).

The Londrina 53 strain was characterized by MLVA and genetic sequencing, and was identified as *L. interrogans* serovar Hardjo genotype Hardjoprajitno (**Figures 2**, **3**). The other two isolated strains (Londrina 49 and Londrina 54) from the same culture medium formulation were molecularly characterized as *L. borgpetersenni* genotype Hardjobovis, which was previously published (Chideroli et al., 2016).

The phylogenetic tree for all isolates shows that the Londrina 53 strain was grouped in the same cluster as *L. interrogans* and had the sequence identity of the serovar Hardjo genotype Hardjoprajitno. The others strains (Londrina 49 and Londrina 54) remained in the same cluster as *L. borgpetersenii* (**Figure 4**).

DISCUSSION

Hardjo is the most prevalent serovar and causative agent of leptospirosis in dairy and beef cattle herds. It causes reproductive

TABLE 2 | Evaluation by dark field microscopy of the cultured Leptospira (Londrina 49, Londrina 53, and Londrina 54 strains) isolated from naturally infected bovine urine.

Evaluation date#	Culture medium*	Dark field microscopic evaluation	Subculture
0 day	A, B, C with antibiotics	NP	NP
1 day	A, B, C with antibiotics	NP	A, B, C without antibiotics
12 ± 2 day	A	Many cells with characteristics and movement similar to Leptospira	A→A A→B A→C
21 ± 2 day	A→C	Few structures with signs of suffering	NP
27 ± 2 day	A→C	Good number of leptospiral cells	A→C→A A→C→B A→C→C
34 ± 3 day	A→C	Stagnation of growth with no moving cells	NP
34 ± 3 day	A→C→B	Many cells similar to Leptospira	A→C→B→B A→C→B→C
41 ± 2 day	A→C→B→B	One Leptospira cell per field	A→C→B→B→B A→C→B→B→C
41 ± 2 day	A→C→B→C	Many cells similar to Leptospira	A→C→B→C→B A→C→B→C→C
48 ± 3 day	A→C→B→C	Good number of leptospiral cells	NP
48 ± 3 day	A→C→B→C→B	Good number of leptospiral cells	A→C→B→C→B→B A→C→B→C→B→C
48 ± 3 day	A→C→B→C→C	Few cells	NP
55 ± 4 day	A→C→B→C→B→B	Optimum growth and adaptation to medium B	A→C→B→C→B→B→B

#On the dark field microscopic evaluation date, all tubes were evaluated, but only the significant data are presented in the table; *Numbers corresponding to the culture media designated in ***Table 1***; → symbol represents a subculture for another medium; NP, not performed.

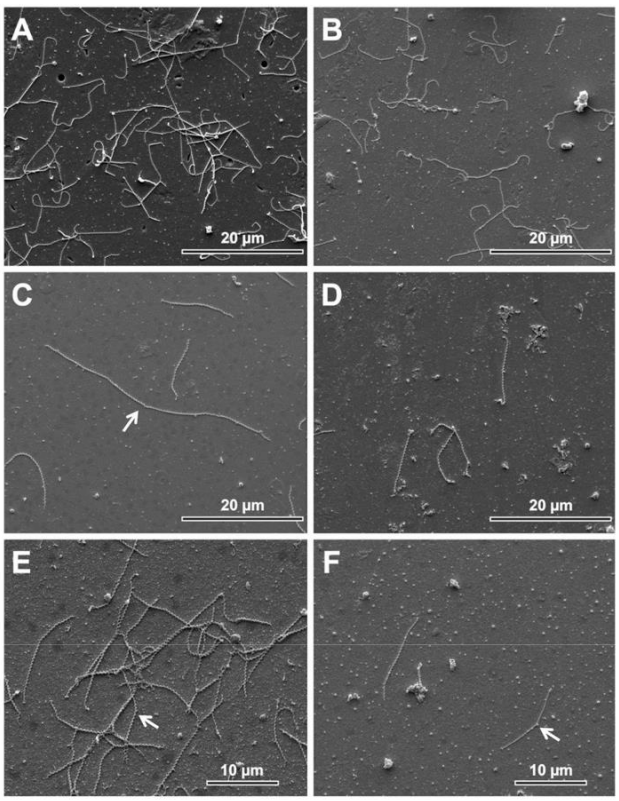

FIGURE 1 | Morphology of leptospire cell in the culture media by scanning electronic microscopy. **(A)** Culture media B with higher number of leptospiral cells of serovar Hardjo; **(B)** Culture media C with fewer number of leptospiral cells of serovar Hardjo; **(C)** culture media B with individualized cells and one elongated cell in division (arrow) of serovar Hardjo. **(D)** Culture media C with fewer cells of Leptospira serovar Hardjo in cell division; **(E)** Presence of typical spirochete morphology in culture media B with cells of Leptospira serovar Hardjo, Arrow—indicates spirochetal corkscrew morphology; **(F)** Lost of typical spirochete morphology in culture media C with cells of Leptospira serovar Hardjo, Arrow—indicates loss of spirochetal corkscrew morphology.

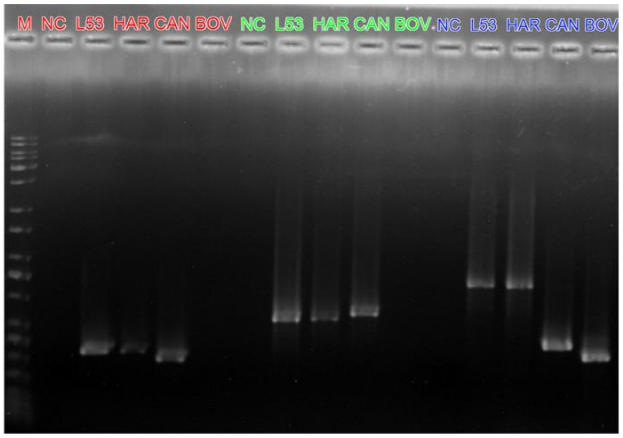

FIGURE 2 | Banding patterns of VNTR visualized in agarose gel. Lane M = 1 kb molecular ladder bp (Kasvi, Curitiba, PR, Brazil), L53 (Londrina 53 strain); HAR = reference sample serovar Hardjo strain Hardjoprajitno; CAN = reference sample serovar Canicola strain Hond Utrecht IV; BOV = reference sample serovar Hardjo strain Hardjobovis; NC = negative control. Locus colors: red (VNTR-4), green (VNTR-7), and blue (VNTR-10).

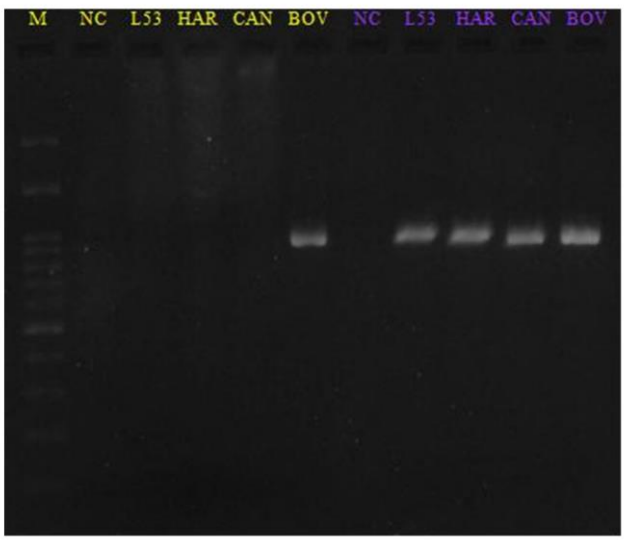

FIGURE 3 | Banding patterns of VNTR visualized in agarose gel. Lane M = molecular ladder bp, L53 (Londrina 53 strain); HAR = reference sample serovar Hardjo strain Hardjoprajitno; CAN = reference sample serovar Canicola strain Hond Utrecht IV; BOV = reference sample serovar Hardjo strain Hardjobovis; NC = negative control. Locus colors: yellow (VNTR-Lb4) and purple (VNTR-Lb5).

failure in livestock worldwide and results in substantial economic loss due to infertility and abortion (Ellis, 2015). In Latin America, few studies have reported recovery of serovar Hardjo (genotype Hardjoprajitno) in cattle (Aycardi et al., 1980; Salgado et al., 2015). In Brazil, two strains, Norma and 2012_OV5, were previously isolated from bovine and ewe, respectively, and characterized as belonging to *L. interrogans* genotype Hardjoprajitno (Cosate et al., 2012; Director et al., 2014). Recently, a Hardjo serovar isolated from bovine urine for the first time in Latin America, was molecularly characterized by MLVA and gene *secY* sequencing as *L. borgpetersenii* genotype Hardjobovis (Chideroli et al., 2016).

EMJH culture media (liquid or semi-solid) is widely used to isolate *Leptospira* (Rodríguez et al., 2002; González et al., 2006; Zacarias et al., 2008; Miraglia et al., 2009; Chakraborty et al., 2011). For many years, our leptospirosis research group has unsuccessfully attempted to isolate serovar Hardjo from bovine urine using unmodified EMJH, even after obtaining positive serology for this serovar (Hashimoto et al., 2017).

In the present study, the combined use of EMJH culture media supplemented with sodium pyruvate (culture medium A); sodium pyruvate and superoxide dismutase (culture medium B); and sodium pyruvate, superoxide dismutase and fetal bovine serum (culture medium C) was used to isolate, adapt, and maintain fastidious leptospires, such as serovar Hardjo, in the laboratory.

The two core ingredients (EMJH medium base and enrichment base) were sustained because they are present in a

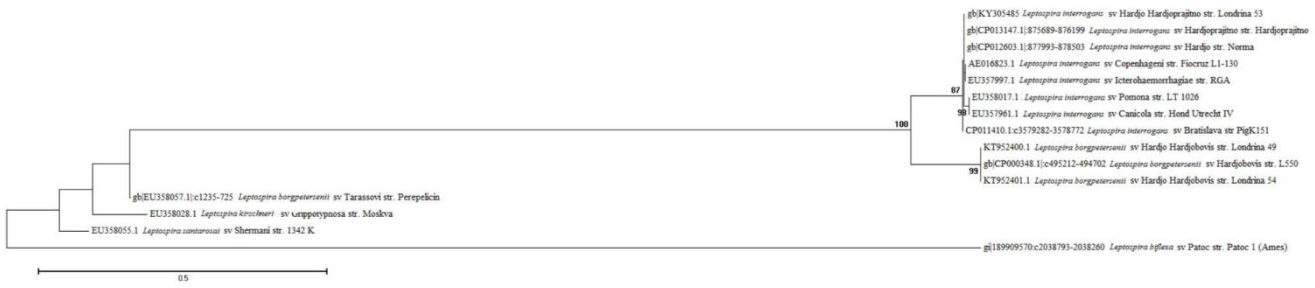

FIGURE 4 | Phylogenetic tree of Leptospira with partial secY gene sequences of the reference strains and a set of clinical isolates from bovine urine (Londrina 49, Londrina 53, and Londrina 54). The phylogenetic tree was drawn using the maximum likelihood method based on the Tamura 3-parameter model. Reliability of the branches was validated by generating 1,000 "bootstrap" replicates. Evolutionary analyses were conducted in MEGA7. The obtained sequences were deposited in the GenBank database with the following access codes: Londrina 49—KT952400, Londrina 53—KY305485, and Londrina 54 – KT952401.

commonly used medium for *Leptospira* isolation, and sodium pyruvate was included in all culture medium formulations because of a previous report that it enhances *Leptospira* growth when added to a solid medium (Johnson et al., 1973). Superoxide dismutase and fetal bovine serum were chosen to aid the growth of *Leptospira* serovar Hardjo, which has exigent nutritional requirements. Different combinations were made to verify the usefulness of each one. More specifically, superoxide dismutase was chosen because it eliminates the toxic radicals produced during spirochete metabolism that accumulate in the culture medium and the concentration was the same used in the culture media for *Treponema* which is a spirochete as well as leptospires (Austin et al., 1981; Cox et al., 1990). Fetal bovine serum was used to replace the rabbit serum present in the enrichment EMJH base (essential requirement for leptospire growth) because serovar Hardjo is thought to be better adapted to the bovine host. Indeed, future studies using a metabolomics approach could help us to better understand the molecules that are involved in adapting these bacteria to the culture media.

These results show that adding these components to the standard culture medium allowed rapid isolation of the Hardjo serovar. As shown in **Table 2**, the formulation with only sodium pyruvate (medium A) was where the first *Leptospira* cells were observed and where the primary isolation occurred. For leptospiral culture media, sodium pyruvate has previously been demonstrated to promote growth (Johnson et al., 1973). In a study on hydrogen peroxide damage in mammalian cell cultures, Giandomenico et al. (1997) found that sodium pyruvate was most effective in eliminating hydrogen peroxide and its toxic effects. Therefore, adding this component to the culture medium for isolating fastidious *Leptospira* serovars may be essential for its primary isolation.

After the first simultaneous subcultures with medium B and C (A→B and A→C), fetal bovine serum was found to be critical for initiating growth. However, when it was passed to a new medium with fetal bovine serum (A→C→C) there was no increase in cell growth. In contrast, subculturing the medium with fetal bovine serum to basal medium supplemented with superoxide dismutase without fetal bovine serum (A→C→B→C→B and A→C→B→C→B→B) increased the bacterial growth and final adaptation in the culture medium. In other words, the fetal bovine serum was important for initial growth but eventually, someway became detrimental, and the culture medium required only superoxide dismutase with an enrichment base of EMJH for final leptospiral adaptation. This result suggests that there is a distinct difference between the culture medium requirement for primary isolation and for the maintenance and adaptation of new isolated strains.

More importantly, the change in leptospiral morphological characteristics in the different media suggests that leptospires in the presence of fetal bovine serum will begin to show signs of suffering such as low growth rate, lower replication rate, loss of their corkscrew-shape and loss of their hooked ends, and will eventually die. A genetics study performed with spirochete non-motile mutants indicate that the periplasmic flagella were involved in spirochete motility. It also indicated that the structure of the flagella influenced the shape of the cell ends (Li et al., 2000). Thus, if the culture medium does not provide the elements necessary for bacterial growth and development, or becomes toxic, the cell metabolism and mobility decrease, and thus, lose their hooked ends (**Figure 1F**). In contrast, the presence of superoxide dismutase seemingly detoxifies the culture medium and allows bacterial development with increased cell size due the possible number of leptospires replicating (**Figure 1C**).

The ability to isolate and maintain *Leptospira* spp. is critical for both diagnostic and research purposes using molecular characterization to identify new isolates (Adler and de la Peña Moctezuma, 2010). To exchange information between laboratories, the MLVA molecular biology technique is efficient, with easy standardization, rapid clinical diagnosis, and can be applied in the field of epidemiology (Salaün et al., 2006; Slack et al., 2006).

Among the techniques used for molecular characterization of leptospiras, the MLST as the MLVA is a simple PCR based technique. The selected loci of MLST are generally the housekeeping genes, which evolve very slowly over an evolutionary time-scale (Enright and Spratt, 1999). However, this methodology depends of sequencing of seven genes which make the technique more expensive and time consuming. In this study, only the *secY* gene was used, which consists of conserved and variable regions with sufficient sequence heterogeneity to enable the phylogenetic classification of *Leptospira* genus (Victoria et al., 2008; Hamond et al., 2016). Another technique widely used for leptospires genotyping is PFGE, but this methodology requires specific structure/equipaments that may not be available in all diagnosis and research laboratories.

Currently, the MLVA method is one practical alternative for differentiation and identification of the many pathogenic *Leptospira* serovar, including the differentiation of two serovar Hardjo genotypes (Salaün et al., 2006). Furthermore, identifying and typing new isolate strains is important for understanding disease epidemiology in the region, as well as developing diagnostic tools, effective vaccines, and prevention strategies for leptospirosis (Ahmed et al., 2011). The results obtained from the MLVA technique were corroborated by the sequential analysis of the partial *secY* gene, which confirmed the genetic species (Ahmed et al., 2006).

CONCLUSION

In this study, culture medium formulations were created to isolate fastidious leptospires of the serovar Hardjo genotypes Hardjobovis and Hardjoprajitno from urine of naturally infected bovine. Noteworthy, additional components were useful for the initial growth (sodium pyruvate and fetal bovine serum) and subsequent maintenance of leptospires (superoxide dismutase) adapted to the medium standard. With this strategy, using three formulations, we succeeded in isolating three pure strains of the serovar Hardjo. After isolation, the technique of MLVA associated with the partial sequencing of gene *secY* have been validated and suggested for molecular characterization of serovars such as Hardjo that may belong to different species. Additionally, an evaluation of leptospire cells in the three formulations by electronic microscopy showed differences in spirochete morphology based on the supplement used in each medium. The superoxide dismutase enzyme induced stretching and cell division; in contrast, cells in the fetal bovine serum medium were fewer in number and lost their corkscrew-shape and hooked ends. Finally, the culture strategies described in this study allowed the isolation and rapid molecular characterization of two serovar Hardjo genotypes inducing new insight into seroepidemiology, specific genotypes, and immunoprophylaxis for leptospirosis in dairy and beef cattle herds.

AUTHOR CONTRIBUTIONS

JdF, UP, and RC planned the project and designed the experiments. RC conducted the experiments and carried out the data analysis with help from JdF, UP, AFA, DG, and AAA. RC, SS, DG, AdO, and UP contributed reagents preparation and samples collection. RC wrote the manuscript, which was critically reviewed by JdF, AAA, AFA, and UP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02155/full#supplementary-material

REFERENCES

Adler, B., Fainea, S., Christophera, W. L., and Chappel, R. J. (1986). Development of an improved selective medium for isolation of leptospires from clinical material. *Vet. Microbiol*. 12, 377–381. doi: 10.1016/0378-1135(86)90087-8

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

Ahmed, A., Thaipadungpanit, J., Boonsilp, S., Wuthiekanun, V., Nalam, K., Spratt, B. G., et al. (2011). Comparison of two multilocus sequence based genotyping schemes for Leptospira species. *PLoS Negl. Trop. Dis*. 5:e1374. doi: 10.1371/journal.pntd.0001374

Ahmed, N., Devi, S. M., Valverde, M. L., Vijayachari, P., Machang'u, R. S., Ellis, W. A., et al. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann. Clin. Microbiol. Antimicrob*. 5:28. doi: 10.1186/1476-0711-5-28

Austin, F. E., Barbieri, J. T., Corin, R. E., Grigas, K. E., and Cox, C. D. (1981). Distribution of superoxide dismutase, catalase, and peroxidase activities among *Treponema pallidum* and other spirochetes. *Infect. Immun.* 33, 372–379.

Aycardi, E. R., Myers, D. M., and Torres, B. (1980). A new Leptospiral serovar in the Tarassovi serogroup from Colombia. *Z. Vet. R. B* 27, 425–428.

Chakraborty, A., Miyahara, S., Villanueva, S. Y. A. M., Saito, M., Gloriani, N. G., and Yoshida, S. I. (2011). A novel combination of selective agents for isolation of *Leptospira* species. *Microbiol. Immunol*. 55, 494–501. doi: 10.1111/j.1348-0421.2011.00347.x

Bey, R. F., and Johnson, R. C. (1978). Protein-free and low-protein media for the cultivation of Leptospira. *Infect*. *Immun*. 19, 562–569.

Chideroli, R. T., Pereira, U. P., Nakamura, A. Y., Alfieri, A. A., Alfieri, A. F., and Freitas, J. C. (2016). Isolation and molecular characterization of *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis in the urine of naturally infected cattle in Brazil. *Genet. Mol. Res.* 15, 1–7. doi: 10.4238/gmr. 15018473

Cosate, M. R. V., Barouni, A. S., Moreira, E. C., Veloso, I. F., Gomes, M. T. R., and Salas, C. E. (2012). Molecular characterization by LSSP-PCR and DNA sequencing of a pathogenic isolate of *Leptospira interrogans* from Brazil. *Zoonoses Public Health* 59, 379–388. doi: 10.1111/j.1863-2378.2012.01470.x

Cox, D. L., Riley, B., Chang, P., Sayahtaheri, S., Tassell, S., and Hevelone, J. (1990). Effects of molecular oxygen, oxidation-reduction potential, and antioxidants upon *in vitro* replication of *Treponema pallidum* subsp. pallidum. *Appl. Environ. Microbiol.* 56, 3063–3072.

de la Peña-Moctezuma, A., Bulach, D. M., Kalambaheti, T., and Adler, B. (1999). Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. *FEMS Microbiol. Lett*. 177, 319–326. doi: 10.1111/j.1574-6968.1999.tb13749.x

Director, A., Penna, B., Hamond, C., Loureiro, A. P., Martins, G., Medeiros, M. A., et al. (2014). Isolation of *Leptospira interrogans* Hardjoprajitno from vaginal fluid of a clinically healthy ewe suggests potential for venereal transmission. *J. Med. Microbiol*. 63, 1234–1236. doi: 10.1099/jmm.0.065466-0

Ellis, W. A. (2015). Animal leptospirosis. *Curr. Top. Microbiol. Immunol*. 387, 99–137. doi: 10.1007/978-3-662-45059-8_6

Ellis, W. A., and Thiermann, A. B. (1986). Isolation of leptospires from the genital tracts of Iowa cows. *Am. J. Vet. Res*. 47, 1694–1696.

Enright, M. C., and Spratt, B. G. (1999). Multilocus sequence typing. *Trends Microbiol*. 7, 482–487. doi: 10.1016/S0966-842X(99)01609-1

Faine, S., Adler, B., Bolin, C., and Perolat, P. (1999). *Leptospira and Leptospirosis, 2nd Edn.* Melbourne, VIC: MedSci Press.

Favero, M., Pinheiro, S. R., Vasconcellos, S. A., Morais, Z. M., Ferreira, F., Neto, J. S. F., et al. (2001). *Leptospirose bovina* - variantes sorológicas predominantes em colheitas efetuadas no período de 1984 a 1997 em rebanhos de 21 estados do Brasil. *Arq. Inst*. *Biológico* 68, 29–35.

Figueiredo, A. D. O., Pellegrin, A. O., Gonçalves, V. S. P., Freitas, B., Monteiro, L. A. R. C., and de Oliveira, J. M. (2009). Prevalência e fatores de risco para a leptospirose em bovinos de Mato Grosso do Sul. *Pesqui. Vet. Bras*. 29, 375–381. doi: 10.1590/S0100-736X2009000500003

Flint, S. H., and Liardet, D. M. (1980). Isolation of *Leptospira interrogans* serovar Hardjo from bovine urine. *N. Z. Vet. J*. 28, 55. doi: 10.1080/00480169.1980.34693

Giandomenico, A. R., Cerniglia, G. E., Biaglow, J. E., Stevens, C. W., and Koch, C. J. (1997). The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide. *Free Radic. Biol. Med*. 23, 426–434. doi: 10.1016/S0891-5849(97)00113-5

González, A., Borrero, R., Ruiz, J., Batista, N., Fernández, Y., Valdés, Y., et al. (2006). Medio EMJH modificado para el cultivo de *Leptospira interrogans* serogrupo Ballum. *Rev. Argent. Microbiol*. 38, 61–68.

Hamond, C., Pestana, C., Medeiros, M., and Lilenbaum, W. (2016). Genotyping of Leptospira directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. *Epidemiol. Infect.* 144, 72–75. doi: 10.1017/S0950268815001363

Hashimoto, V. Y., Chideroli, R. T., Ribeiro, J., Alfieri, A. A., Costa, G. M., Freitas, J. C., et al. (2017). Serological and molecular findings in diagnosis of leptospirosis serovar hardjo in a dairy bovine herd. *Semin. Cien. Agrar.* 38, 3155–3164. doi: 10.5433/1679-0359.2017v38n5p3155

Hashimoto, V. Y., Dias, J. A., Spohr, K. A. H., Silva, M. C. P., Andrade, M. G. B., Müller, E. E., et al. (2012). Prevalência e fatores de risco associados

á *Leptospira* spp. em rebanhos bovinos da região centro-sul do estado do Paraná. *Pesqui. Vet. Bras*. 32, 99–105. doi: 10.1590/S0100-736X20120002 00001

Herrmann, J. L., Bellenger, E., Perolat, P., Baranton, G., and Saint Girons, I. (1992). Pulsed-field gel electrophoresis of NotI digests of leptospiral DNA: a new rapid method of serovar identification. *J. Clin*. *Microbiol*. 30, 1696–1702.

Johnson, R. C., and Gary, N. D. (1962). Nutrition of Leptospira pomona. 1. A chemically defined substitute for rabbit serum ultrafiltrate. *J. Bacteriol.* 83, 668–672.

Johnson, R. C., and Rogers, P. (1964). 5-Fluorouracil as a selective agent for growth of Leptospirae. *J. Bacteriol*. 87, 422–426.

Johnson, R. C., Walby, J., Henry, R. A., and Auran, N. E. (1973). Cultivation of parasitic leptospires: effect of pyruvate. *Appl*. *Microbiol*. 26, 118–119.

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Leonard, F. C., Quinn, P. J., Ellis, W. A., and O'Farrell, K. (1992). Duration of urinary excretion of leptospires by cattle naturally or experimentally infected with *Leptospira interrogans* serovar hardjo. *Vet. Rec*. 131, 435–439. doi: 10.1136/vr.131.19.435

Li, C., Motaleb, A., Sal, M., Goldstein, S. F., and Charon, N. W. (2000). Spirochete periplasmic flagella and motility. *J. Mol. Microbiol*. *Biotechnol*. 2, 345–354.

Majed, Z., Bellenger, E., Postic, D., Pourcel, C., Baranton, G., and Picardeau, M. (2005). Identification of variable-number tandem-repeat loci in *Leptospira interrogans* sensu stricto. *J. Clin. Microbiol*. 43, 539–545. doi: 10.1128/JCM.43.2.539-545.2005

Miraglia, F., De Moraes, Z. M., Melville, P. A., Dias, R. A., and Vasconcellos, S. A. (2009). Emjh medium with 5-fluorouracil and nalidixic acid associated with serial dilution technique used to recover leptospira spp from experimentally contaminated bovine semen. *Braz. J. Microbiol*. 40, 189–193. doi: 10.1590/S1517-83822009000100033

Myers, D. M. (1975). Efficacy of combined furazolidone and neomycin in the control of contamination in Leptospira cultures. *Antimicrob. Agents Chemother*. 7, 660–671. doi: 10.1128/AAC.7.5.666

Pailhoriès, H., Buzelé, R., Picardeau, M., Robert, S., Mercier, E., Mereghetti, L., et al. (2015). Molecular characterization of Leptospira sp by multilocus variable number tandem repeat analysis (MLVA) from clinical samples: a case report. Int. J. Infect. Dis. 37, 119–121. doi: 10.1016/j.ijid.2015.06.026

Perolat, P., Lecuyer, I., Postic, D., and Baranton, G. (1993). Diversity of ribosomal DNA fingerprints of Leptospira serovars provides a database for subtyping and species assignation. Res. Microbiol. 144, 5–15. doi: 10.1016/0923-2508(93)90210-S

Picardeau, M. (2013). Diagnosis and epidemiology of leptospirosis. Med. Mal. Infect. 43, 1–9. doi: 10.1016/j.medmal.2012.11.005

Pourcel, C., Vidgop, Y., Ramisse, F., Vergnaud, G., and Tram, C. (2003). Characterization of a tandem repeat polymorphism in Legionella pneumophila and its use for genotyping. J. Clin. Microbiol. 41, 1819–1826. doi: 10.1128/JCM.41.5.1819-1826.2003

Ralph, D., McClelland, M., Welsh, J., Baranton, G., and Perolat, P. (1993). Leptospira species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. J. Bacteriol. 175, 973–981. doi: 10.1128/jb.175.4.973-981.1993

Robertson, A., Boulanger, P., and Mitchell, D. (1964). Isolation and identification of a leptospire of the hebdomadis serogroup (L hardjo) from cattle in Canada. Can. J. Comp. Med. 28, 13–18.

Rodríguez, A. G., Santiesteban, N. B., Abreu, Y. V., and González, M. G. (2002). Crecimiento, virulencia y antigenicidad de Leptospira interrogans serovar mozdok en medio EMJH modificado. Rev. Cuba. Med. Trop. 54, 32–36.

Salaün, L., Mérien, F., Gurianova, S., Baranton, G., and Picardeau, M. (2006). Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. J. Clin. Microbiol. 44, 3954–3962. doi: 10.1128/JCM.00336-06

Salgado, M., Otto, B., Moroni, M., Sandoval, E., Reinhardt, G., Boqvist, S., et al. (2015). Isolation of Leptospira interrogans serovar Hardjoprajitno from a calf with clinical leptospirosis in Chile. BMC Vet. Res. 11:66. doi: 10.1186/s12917-015-0369-x

Silva, F. J., Conceição, W. L. F., Fagliari, J. J., Girio, R. J. S., Dias, R. A., Borba, M. R., et al. (2012). Prevalência e fatores de risco de leptospirose bovina no Estado do Maranhão. Pesqui. Vet. Bras. 32, 303–312. doi: 10.1590/S0100-736X2012000400006

Slack, A. T., Symonds, M. L., Dohnt, M. F., and Smythe, L. D. (2006). Identification of pathogenic Leptospira species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. BMC Microbiol. 6:95. doi: 10.1186/1471-2180-6-95

Staneck, J. L., Henneberry, R. C., and Cox, C. D. (1973). Growth requirements of pathogenic leptospira. *Infect*. *Immun*. 7, 886–897.

Thiermann, A. B., Handsaker, A. L., Moseley, S. L., and Kingscote, B. (1985). New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar kennewicki. *J. Clin. Microbiol*. 21, 585–587.

Victoria, B., Ahmed, A., Zuerner, R. L., Ahmed, N., Bulach, D. M., and Hartskeerl, R. A. (2008). Conservation of the S10-spc- a locus within otherwise highly plastic genomes provides phylogenetic insight into the genus Leptospira. *PLoS ONE* 3:e2752. doi: 10.1371/journal.pone.0002752

Wuthiekanun, V., Amornchai, P., Langla, S., Oyuchua, M., Day, N. P. J., and Limmathurotsakul, D. (2014). Maintenance of *Leptospira* species in Leptospira Vanaporn Wuthiekanun agar. *J. Clin. Microbiol*. 52, 4350–4352. doi: 10.1128/JCM.02273-14

Zacarias, F. G. D. S., Vasconcellos, S. A., Anzai, E. K., Giraldi, N., De Freitas, J. C., and Hartskeerl, R. (2008). Isolation of Leptospira serovars Canicola and Copenhageni from cattle urine in the State of Parana, Brazil. *Braz. J. Microbiol*. 39, 744–748. doi: 10.1590/S1517-83822008000400028

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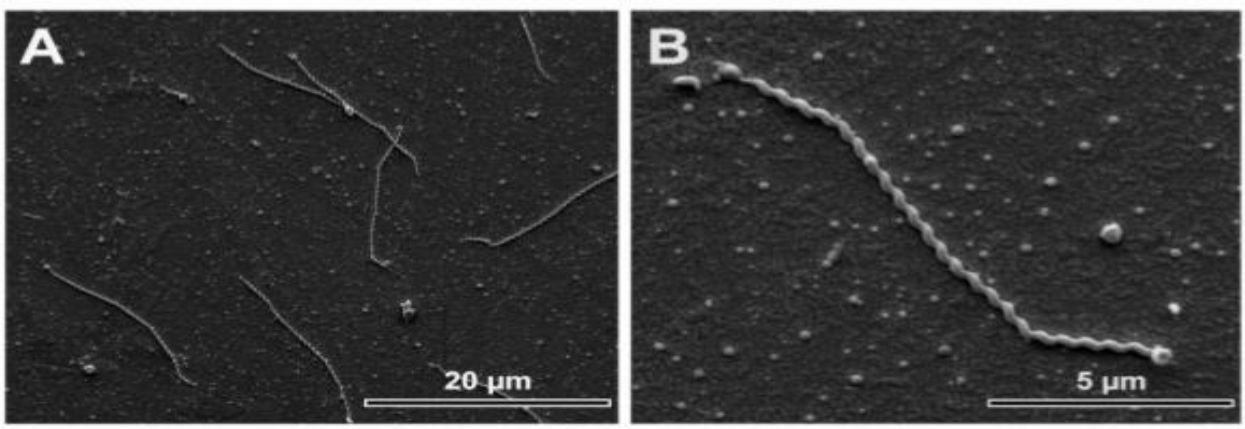

Supplementary Figure 1: Morphology of leptospire cell in the culture media A by electronic microscopy. (A) Example of a standard *Leptospira* culture with 7 days of growth; **(B)** Single cell of *Leptospira* serovar Hardjo showing details of the spirochete morphology.

7. ARTIGO B - Genomics approaches to animal leptospirosis control: screening of vaccine candidates and new drugs

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Abstract

Leptospirosis is an emerging disease of worldwide relevance, however, there is still a limitation in its control due to short time immunogenic vaccines for animals and no-existence for humans. Also, studies related to treatment are required, especially in asymptomatic animals, renal carriers. Microbial genome analysis can contribute to provide new approaches for the treatment and control of leptospirosis. In this study, the genome of two serovar Hardjo were performed, and compared with nineteen genomes from isolates of most pathogenic to animals (*L. interrogans* and *L. borgpetersenii*). First, comparative genomics showed quite a similarity between strains of the same species. Additionally, it identified islands of pathogenicity to study if any vaccine or drug targets are present in these islands. Then, through bioinformatics tools and a subtractive genomics approach, the core genome of these species was analyzed to search for new drug and vaccine targets. The OrthoFinder software found a total of two hundred and twenty-one genes in the core genome with no homology to animal or human host. After the characterization and subcellular localization of the proteins, these genes were directed to reverse vaccinology and search for new drug targets with molecular docking. Seventeen proteins were identified as good candidate vaccine target and classified according to structures, its adhesion capacity to MHC-I and MHC-II and epitope densities, and of these, two were present in islands of pathogenicity. Taking into account the characteristics of adhesin probability, virulence, and epitope density, five targets may be good candidates. For the molecular docking, eight proteins predicted as cytoplasmic and considered essential for the survival of leptospires were considered good targets for new drugs. Highlighting for the cell division protein FtsZ, which was the target identified with the best binding characteristics and the one drug was identified as the best molecule for linked to this protein in order to inhibit its

function and consequently harm the leptospire multiplication. All the data highlight here has great relevance for public and animal health, as it finds new therapeutic and vaccine targets which may be tested quickly for leptospirosis prevention representing a breakthrough in the field.

1 INTRODUCTION

Leptospirosis is an emerging zoonosis, especially in Central and South America, which impacts both human and animal health worldwide and is caused by pathogenic bacteria of the genus *Leptospira* (Costa et al., 2019). Two classifications are used for leptospires to identify the species, one determined by serology, with the serovar as the basic taxon, and another established according to their molecular characteristics, recognized to as genomospecies (Adler and de la Peña Moctezuma, 2010). Although many species infect animals, *Leptospira interrogans* and *L. borgpetersenii* are the two major species that contain the serovars most frequently found causing disease in pet and farm animals. Additionally, In host-adapted conditions, cattle, dogs, and small rodents can act as maintenance hosts for Hardjo, Canicola, and Icterohaemorrhagiae serogroups, respectively (Ellis, 2015).

Farms animals are susceptible to clinical infection, resulting in economic losses due to frequent abortions, decreased milk production, reproductive failure, premature birth or stillbirth (Martins and Lilenbaum, 2013; Pinto et al., 2017). Dogs are more susceptible and the clinical symptoms may be variable depending on infecting serovar, with asymptomatic animals or with mild disease, and with severe and life-threatening presentation (Lee et al., 2014). After infection, and even without clinical signs, these animals remain mostly chronic renal carriers and continue to eliminate leptospires through the urine in the environment (Ellis, 2015; Paes et al., 2016). Clinicians often report the failure of antibiotic therapy in the leptospiruria phase, with detection of DNA or isolation of *Leptospira* from urine samples, even after a long period of antibiotic therapy in both animals and humans (Daher et al., 2012; Ratet et al., 2014).

Due to mechanisms involved in the ability of leptospires to survive in the kidneys, such as biofilm formation, few drugs are effective in cleaning leptospires from the renal tubules (Ratet et al., 2014). Considering that the drug features are a pivotal factor in the success of treatment and there is evidence of limited efficacy of commonly used drugs, the discovery of new drugs alternative to the use of these antimicrobials are imperative. A rational use of antimicrobials, as well as, studies aimed at the discovery of new drugs or products alternative to the use of antimicrobials are imperative (Kraemer et al., 2019).
Vaccination is the best strategy for leptospirosis control and particularly to reduce cases of renal carriers in farmed animals and dogs (Adler and de la Peña Moctezuma, 2010). Current vaccines had few innovations strategies in the last decades. Those based on inactivated whole-cell preparations (bacterins) still are commercially available, even though in the last decade a search for a recombinant vaccine for human leptospirosis has started (Adler and de la Peña Moctezuma, 2010; Dellagostin et al., 2011). However, in the meantime, there has been an increase and improvement of molecular techniques associated with the use of bioinformatics tools (Rappuoli et al., 2016).

Reverse vaccinology, prediction of subcellular localization, and molecular docking should be considered an alternative since they optimize the prediction of drug and vaccine targets, especially for fastidious microorganisms, such as *Leptospira* sp (Zeng et al., 2017). Besides, simultaneous analysis of targets across multiple genomes can be very useful for greater coverage among multiple serovars (Dellagostin et al., 2017).

In this context, the objective of this work was to accomplish the sequencing and assembly of two new isolates of the serovar Hardjo of the genotype Hardjobovis (L49) and Hardjoprajitno (L53) and to predict drug and vaccine targets through bioinformatics tools searching in this two strains and more 19 strains of two main *Leptospira* species for animals (*L. interrogans* and *L. borgpetersenii*) whose genomes are deposited in the GenBank. This study will collaborate with future in vitro and in vivo tests for the development of new drugs and vaccine targets against this pathogen etiologically related to a major global zoonosis.

2 MATERIALS AND METHODS

2.1 Sequencing and assembly of strains genomes

Genome sequencing of the strains L49 and L53 was performed with the MiSEQ platform (Illumina®, USA), using a 300-bp paired-end-library. Reads were uploaded in FASTQ format to CLC Genomics Workbench 11 (Qiagen, USA) software, for trimming and assembly steps according to Facimoto et al., 2018. In order to build the scaffold draft of the genome, contigs were ordered through CONTIGuator 2.7 software (Galardini et al., 2011), using the *Leptospira interrogans* serovar Hardjo str. Norma and *Leptospira borgpetersenii* serovar Hardjo str. L550 as a reference for L53 and L49, respectively. All the raw sequencing data was mapped in the final genome sequence and the absence of contamination with other genomes was confirmed by the coverage and the low number of unmapped reads (less than 0.1%). Automatic structural and functional annotation (to predict genes, rRNAs, and tRNAs) of the genome was performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP).

2.2 Comparative genomic and phylogeny reconstruction

Two inclusion criteria were used for genome selection: *Leptospira* species important in animal pathogenesis and having more than two complete genomes available in the GenBank database. Thus, 15 strains of *L. interrogans* and six of *L. borgpetersenii* available were downloaded in FASTA format through the National Center for Biotechnology Information (NCBI) for the bioinformatics analysis. The general information of all genomes used in this study can be found in Supplementary Table 1.

The 21 genomes (plus another genome of a non-pathogenic species *L. biflexa*) were submitted to Gegenees tool to compare the whole genomes, all against all, and generate a heatmap showing the differences and similarity between each strain of *L. interrogans* and *L. borgpetersenii* (Ågren et al., 2012). This comparative analysis gives phylogenomic overviews, and define genomic signatures unique to specified target groups. The results were exported in the NEXUS format for later phylogenetic reconstruction using the software SplitsTree4 by the neighbor-joining method (Huson and Bryant, 2006). These analyses were performed with two chromosomes of each genome.

After, 13 genomes of phylogenetically closest serovars according to the SplitsTree program were arranged into clusters for prediction analyses of pathogenicity islands (PAI) using GIPSy software (Soares et al., 2016). The genome of the species *L. biflexa* was selected as a reference in predicting the islands because is not pathogenic to humans or animals. In sequence, to display the selected targets in the predicted genomic islands, a figure of ring alignment was generated using BRIG software (Alikhan et al., 2011). In addition to the prediction of the PAI, an analysis was included using Virulence Factor Database (VFDB) for

the search for targets with virulence properties that enable a microorganism to install itself on or within a host and improve its potential to cause infection (Liu et al., 2019). Thus, it was considered a potential if the target gave a match of similarity equal to or greater than a cut-off of 30 in the bit score.

2.3 Identification of conserved proteins of *L. interrogans* and *L. borgpetersenii* and subtractive genomics

To identify regions of homology and conserved proteins between the 21 leptospiras genomes, FASTA format files containing the amino acid (aa) sequences were submitted to the software OrthoFinder under its default parameters. In this analysis, the genes were grouped into: core genes, which are present in all evaluated strains; shared genes, which are present in some, but not all strains; and singletons, which are specific genes present in only one strain (Emms and Kelly, 2019).

In this step, the files were submitted to this software several times, following two strategies. First, *L. interrogans* genomes from strains isolated from human and animal hosts were submitted separately; and then this same procedure for *L. borgpetersenii* strains. This first strategy resulted in four core genes*: L. interrogans* isolates from humans and other isolates from animals; *L. borgpetersenii* isolates from humans, and other isolates from animals. Subsequently, steps were taken in order to identify proteins belonging to leptospires but without homology to human or animal hosts. Thus, the core proteins were subjected to BLASTp searches, also using OrthoFinder, to identify only proteins belonging to the bacteria without homology human genome. For the animal host (cattle, canines, mice, swine and rats), a BLASTp was performed against the proteins of these animal genomes, but using the NCBI GenBank database. This plane is known as subtractive genomics and was necessary to avoid the selection of drug or vaccines targets without protective effect or even those that may induce autoimmunity (Mondal et al., 2015). Then, as a second strategy, the resultant core genes of the previous step (including non-host-homology) were submitted again to the OrthoFinder software to obtain a final core of the two *Leptospira* species.

2.4 Prediction of the subcellular location and characterization of proteins

The classification of proteins (cytoplasmatic, membrane, secreted, and putatively exposed to the surface -PSE) in order to organize and separate the proteins according to their subcellular location was performed by the SURFG software using markers such as identification of peptide signals, retention signals, transmembrane helices, and secretion pathways in the amino acid sequence. Thus, only proteins recognized as cytoplasmic were subjected to an investigation of possible drug targets, while proteins characterized as membrane, PSE, and secreted were led to reverse vaccinology analysis. (Barinov et al., 2009).

In the next step, we analyzed which protein targets were really essential for the microorganism's metabolism. For this strategy, we searched the database of essential genes (DEG) following the criteria of bit score of 100 and e-value with a cut-off of $1 \times 10{-}4$. This online platform (http://www.essentialgene.org/) gives information on bacteria, archaea, and eukaryotic genes, as well as data from non-coding RNAs (Zhang et al., 2004).

2.5 Selection of vaccine targets

All proteins previously selected for vaccine-targets were evaluated for their adhesion ability

to the main histocompatibility complex (MHC) class I and class II, using the Vaxign tool (http://www.violinet.org/vaxign /). This vaccine design program predicts targets, using the reverse vaccinology strategy with standard parameters considering a cutoff point of 0.51 for the adherence probability. Reverse vaccinology also includes the identification and evaluation of epitopes using the Vaxitop program of the Vaxign tool and searches into the Immune Epitope Database with a threshold of 0.5 (IEDB) (He et al., 2010).

In the platform, it is possible to find the main epitopes and the value of each residue (Vita et al., 2019). Epitopes with fewer than seven aa were discarded from the study because they are considered too small to induce immunogenicity. Finally, the B-cell epitope density was calculated by dividing the number of predicted linear B-cell epitopes by protein length (Santos et al., 2013).

2.6 Analysis of proteins of interest

In summary, we used the Universal Protein Resource (UniProt), which is a comprehensive resource for protein sequence and annotation data (UniProt Consortium, 2008). The SignalP program was used to identify the proteins that had signal peptide, which located the cleavage sites of each signal peptide (Petersen et al., 2011). To predict transmembrane helices, we submitted the faa sequences protein to the TMHMM server, which predicted the topology of these proteins by the Markov method (Krogh et al., 2001).

Another strategy is to investigate previous studies of potential drug-targets by searching in the DrugBank database. In this online database, we find comprehensive information about drugs, their targets and clinical trials in progress and the completed ones (Wishart et al., 2018).

2.7 Selection of drug targets and docking analysis

Three-dimensional model structures of cytoplasmatic proteins were predicted by MHOLline software (http://www.mholline2.lncc.br/) using Protein Data Bank (PDB) homology. This tool combines other programs such as HMMTOP, BLAST, BATS, MODELLER, and PROCHECK to analyze potential drug targets according to their structural quality and generate the 3D protein model (Capriles et al. 2010). Only models with identity $\geq 50\%$ and an e-value ≤ 0.3 (good to very high-quality sequences, according to MHOLline score) were submitted to the next stages of docking.

Another step in the subtractive in silico approach was druggability analyzes. For this, the final lists of the drug's target proteins (.pdb format) were submitted to the DoGSiteScorer tool, available on the ProteinPlus server (https://proteinsplus.zbh.uni-hamburg.de). From 3D models of the selected proteins, attachment sites, designated pockets, were identified, where the amino acids denominated residues were identified as the best docking site with the ligand molecule (Volkamer et al., 2012). For each detected cavity, the program returns the pocket residues and a probability score ranging from 0 to 1. Values closer to 1 indicate highly druggable protein cavities, wherein the ligand fits with high affinity (Jamal et al., 2017). The proteins with druggable cavity presenting a value score greater than 0.8 were selected to further analysis. The DoGSiteScorer also calculates volume, surface area, and other related parameters for each predicted cavity. After that, residues were in most favored regions were evaluated using the Ramachandran plot through the SAVES v5.0 server.

The library of binding compounds was obtained from the ZINC database by the download in SDF format of the natural products category and its derivatives (Irwin and Shoichet, 2005).

The ZINC database compounds and protein targets were converted to PDBQT format using OpenBabel and AutoLockTools MGL tools to be manipulated in the Autodock Vina program in the final docking stage (O'Boyle et al., 2011; Morris et al., 2010). For a virtual screening, a grid box parameter was created for each target, comprising residues from the druggable pocket using the AutoDock Vina tool (Trott and Olson, 2010). Initially, the screening evaluated all the ligands (5008) in the library and then, the top 10 classified ligand molecules were identified by a new virtual screening using a Python script. The flexible docking with 3D poses was performed only with these top 10 molecules using the Chimera program to visualize the target-ligand bond (Pettersen et al., 2004).

3 Results and discussion

The methodologies used in the most important steps to identify vaccine and drug targets, as well as, the total number of proteins described in each step is summarized in the workflow of Figure 1.

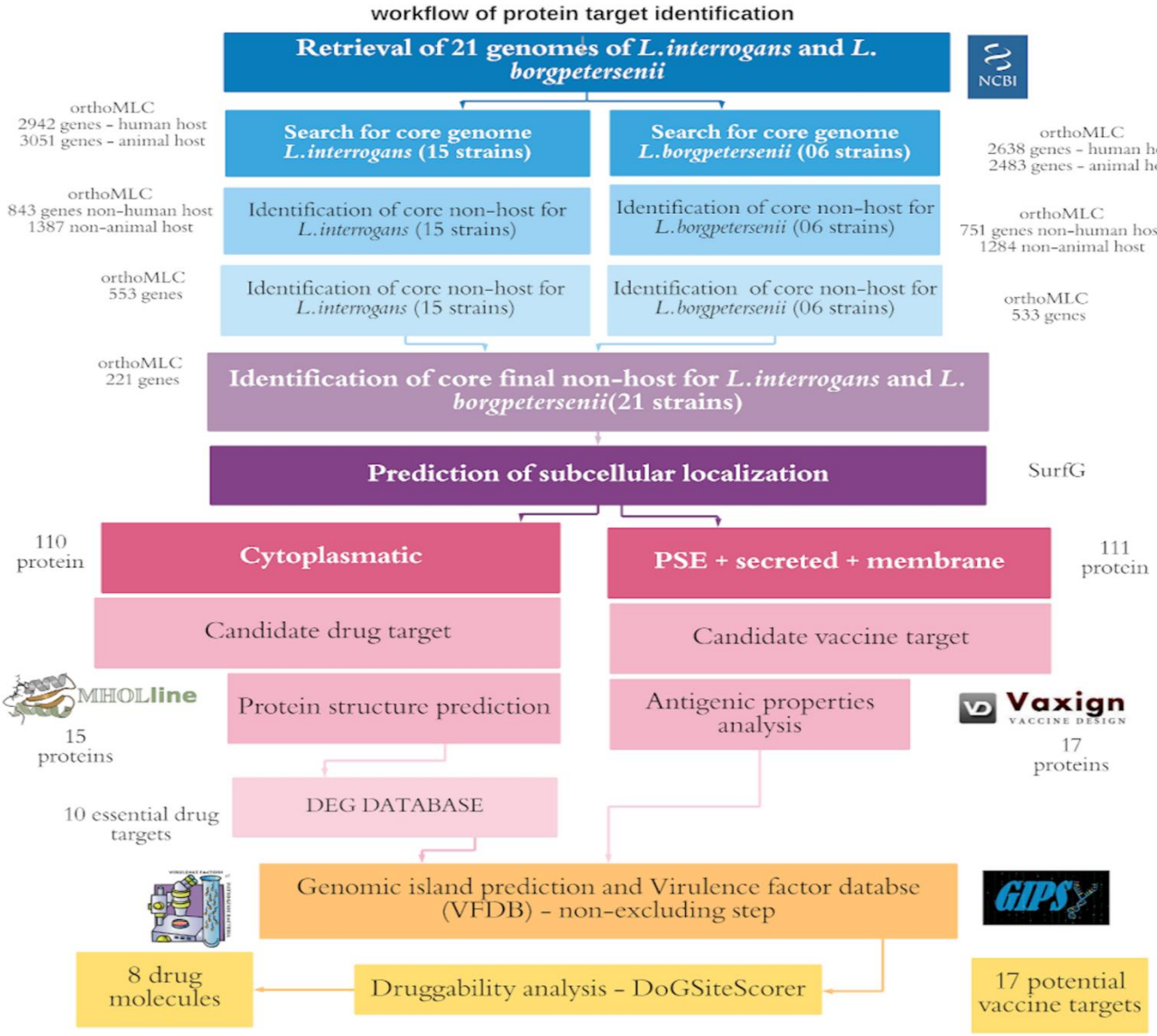

Figure 1. Workflow designed with the methodologies used and the total number of proteins identified in each step.

3.1 Comparative genomic analysis and phylogenetic reconstruction

Leptospira is a genus of bacteria with complex pathogenicity and contains different species and serovars (Vincent et al., 2019), so we decided to carry out comparative genomics analyses to study the relationship between the strains of two species.

The genomes studied showed a high similarity when comparing strains of the same species (Figure 2). Most genomes presented around 90% similarity, with the lowest being 80%. On the other hand, when compared *L. interrogans* with *L. borgpetersenii* strains, there is a low (<17%) similarity. Interestingly, in the chromosome II heat map (Figure 3), the *L. interrogans* serovar Bratislava strain PigK151 showed very low similarity with both species suggesting that it should be more explored for better knowledge about the taxonomy of this strain or review its genome sequencing for any assembly errors in chromosome II.

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1: L.borgpetersenii_ser_Ballum_strain_56604_chromosome_I	100	96	85	85	85	86	0	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13
2: L.borgpetersenii_ser_Ceylonica_strain_Piyasena_chromosome_I	94	100	84	84	84	85	0	13	13	13	13	13	13	14	13	14	14	13	13	13	14	14
3: L.borgpetersenii_ser_Hardjo_strain_JB197_chromosome_I	80	81	100	92	92	92	0	16	15	15	14	14	14	14	14	15	15	15	14	14	14	14
4: L.borgpetersenii_ser_Hardjo_strain_L49_chromosome_I	86	87	99	100	100	100	0	17	15	15	15	15	14	15	15	15	15	16	14	14	14	14
5: L.borgpetersenii_ser_Hardjo_strain_203_chromosome_I	86	87	99	100	100	100	0	16	15	15	15	15	14	15	15	16	16	16	15	15	15	15
6: L.borgpetersenii_ser_Hardjo_strain_L550_chromosome_I	87	88	98	99	100	100	0	16	15	15	15	15	14	15	15	16	16	16	15	15	15	15
7: L.biflexa_ser_Patoc_strain_Paris_chromosome_I	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8: L.interrogans_ser_Hardjo_strain_Norma_chromosome_I	10	10	12	11	11	11	0	100	91	84	83	82	84	85	84	84	84	90	81	81	81	81
9: L.interrogans_ser_Bratislava_strain_PigK151_chromosome_I	10	10	11	10	10	10	0	92	100	85	84	84	85	86	85	85	85	84	82	82	82	82
10: L.interrogans_strain_FMAS.AW1_chromosome_I	11	11	11	11	11	11	0	89	90	100	92	91	90	89	89	90	90	89	87	87	87	87
11: L.interrogans_strain_FMAS.KW2_chromosome_I	11	12	11	11	11	11	0	91	92	94	100	97	92	91	91	92	92	91	90	90	89	89
12: L.interrogans_strain_FMAS.KW1_chromosome_I	11	11	11	11	11	11	0	91	92	95	98	100	93	92	91	92	92	91	90	90	90	90
13: L.interrogans_ser_Linhai_strain_56609_chromosome_I	11	11	11	11	11	11	0	93	93	93	93	92	100	93	93	93	93	92	91	91	89	89
14: L.interrogans_ser_Lai_strain_IPAV_chromosome_I	11	11	11	11	11	11	0	93	93	92	91	91	92	100	100	95	95	92	90	90	90	90
15: L.interrogans_ser_Lai_strain_56601_chromosome_I	11	11	11	11	11	11	0	93	93	92	91	91	92	100	100	95	95	92	90	90	90	90
16: L.interrogans_ser_Copenhageni_str_Fiocruz_L1.130_chromosome_I	11	12	11	11	11	11	0	93	94	93	92	92	93	96	96	100	100	93	91	91	91	91
17: L.interrogans_ser_Copenhageni_strain_FDAARGOS_203_complete	11	12	11	11	11	11	0	93	94	93	92	92	93	96	96	100	100	93	91	91	91	91
18: L.interrogans_ser_Hardjo_strain_L53_chromosome_I	11	11	12	12	12	12	0	100	92	92	91	91	92	93	93	93	93	100	89	89	89	89
19: L.interrogans_ser_Canicola_strain_611_chromosome_I	11	12	12	11	11	12	0	90	91	91	91	90	91	91	91	92	92	89	100	100	89	89
20: L.interrogans_ser_Canicola_strain_LJ178_chromosome_I	11	12	12	12	12	12	0	90	90	91	91	90	91	91	91	92	92	89	100	100	89	89
21: L.interrogans_ser_Manilae_strain_UPMMCNIIDLP_chromosome_I	11	12	11	11	11	11	0	90	90	91	90	90	90	92	91	92	92	90	89	89	100	100
22: L.interrogans_ser_Manilae_strain_UPMMCNIIDHP_chromosome_I	11	12	11	11	11	11	0	90	90	91	90	90	90	92	91	92	92	90	89	89	100	100

Figure 2. Heatmap shows a similarity between the genomes of *Leptospira* spp. (chromosome I) with colors varied in a scale from green to red, equivalent to the percentages of similarity existing in the best-aligned fragments.

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1: L.borgpetersenii_ser_Ballum_strain_56604_chromosome_II	100	97	87	87	86	84	0	5	10	10	10	10	11	11	12	11	10	10	11	10	10
2: L.borgpetersenii_ser_Ceylonica_strain_Piyasena_chromosome_II	95	100	85	85	84	81	0	3	10	10	10	10	10	11	11	11	10	10	10	10	10
3: L.borgpetersenii_ser_Hardjo_strain_203_chromosome_II	88	88	100	100	99	94	0	5	10	10	10	10	10	10	12	12	10	10	10	10	10
4: L.borgpetersenii_ser_Hardjo_strain_L550_chromosome_II	88	88	100	100	99	94	0	5	10	10	10	10	10	10	12	12	10	10	10	10	10
5: L.borgpetersenii_ser_Hardjo_strain_L49_chromosome_II	88	88	100	100	100	94	0	5	10	10	10	10	10	10	12	12	10	10	10	10	10
6: L.borgpetersenii_ser_Hardjo_strain_JB197_chromosome_II	89	89	99	99	98	100	0	5	10	10	10	10	10	10	12	12	10	10	10	10	10
7: L.biflexa_ser_Patoc_strain_Paris_chromosome_II	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8: L.interrogans_ser_Bratislava_strain_PigK151_chromosome_II	2	2	2	2	2	2	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0
9: L.interrogans_ser_Manilae_strain_UPMMCNIIDLP_chromosome_II	8	8	9	9	9	9	0	2	100	100	91	91	92	93	93	92	92	92	92	91	92
10: L.interrogans_ser_Manilae_strain_UPMMCNIIDHP_chromosome_II	8	8	9	9	9	9	0	2	100	100	91	91	92	93	93	92	92	92	92	91	92
11: L.interrogans_ser_Canicola_strain_611_chromosome_II	8	8	8	8	8	8	0	1	92	92	100	100	95	93	93	93	93	93	93	92	92
12: L.interrogans_ser_Canicola_strain_LJ178_chromosome_II	9	9	9	9	9	9	0	1	92	92	100	100	95	93	93	93	93	93	93	92	92
13: L.interrogans_ser_Linhai_strain_56609_chromosome_II	10	9	9	9	9	9	0	1	92	92	94	94	100	95	95	95	95	95	95	94	95
14: L.interrogans_strain_FMAS.KW1_chromosome_II	9	9	9	9	9	9	0	1	93	93	93	93	95	100	99	97	95	95	95	94	95
15: L.interrogans_strain_FMAS.KW2_chromosome_II	10	10	10	10	10	10	0	2	92	92	92	92	95	98	100	97	94	94	95	94	94
16: L.interrogans_strain_FMAS.AW1_chromosome_II	9	9	9	9	9	8	0	2	92	92	92	92	95	96	96	100	95	95	95	94	94
17: L.interrogans_ser_Lai_strain_IPAV_chromosome_II	9	9	9	9	9	8	0	1	92	92	92	92	96	95	94	96	100	100	96	95	95
18: L.interrogans_ser_Lai_strain_56601_chromosome_II	9	9	9	9	9	8	0	1	92	92	92	92	96	95	94	95	100	100	96	95	95
19: L.interrogans_ser_Hardjo_strain_Norma_chromosome_II	10	9	9	9	9	8	0	1	93	93	93	93	96	95	95	96	96	96	100	98	96
20: L.interrogans_ser_Hardjo_strain_L53_chromosome_II	9	9	9	9	9	8	0	1	93	93	93	93	96	96	95	96	97	97	99	100	97
21: L.interrogans_ser_Copenhageni_str_Fiocruz_L1.130_chromosome_II	9	9	9	9	9	8	0	1	94	94	94	94	96	96	96	97	97	97	98	97	100

Figure 3. Heatmap shows a similarity between the genomes of *Leptospira* spp. (chromosome II) with colors varied in a scale from green to red, equivalent to the percentages of similarity existing in the best-aligned fragments.

3.2 Prediction of genomic island

To predict genomic islands, we selected 13 genomes belonging to the different serovars strains of the *L. interrogans* and *L. borgpetersenii* observed in the clusters on phylogenetic analyses. To view these results, the BRIG software was used, which it is possible to observe colors rings that represent each genome and the nucleotide similarity that exists between the genomic sequences and the reference. The reference genome used was *L. borgpetersenii* Hardjobovis strain L49 with its 17 pathogenic islands identified by the Gipsy software. This similarity can be observed by the intensity of the colors of the rings, which increases or decreases according to the identity obtained by BLAST. About the virulence feature, among the vaccine targets, the WP_011670486 protein was predicted within PAI9 and the WP_011670733 protein within PAI14. The other proteins indicated as vaccine targets were not found in regions of pathogenicity islands. In the meanwhile, no potential drug targets were observed within any PAI (Figure 4).

These data reveal that despite the similarity between strains of the same species, there is a significant difference between strains of different species. This seems to be an important obstacle, since the main objective of this work was to find potential core targets for drugs and vaccines that act against all strains of both species which certainly is a notable strategy. Since the main objective of this work was to find potential targets for drugs and vaccines that act against all strains of both species. A large number of species and serovars of leptospires and difference between genomes may be the reason for not having a validated universal vaccine

yet (Conrad et al., 2017; Dellagostin et al., 2017; Dellagostin et al., 2011; Grassmann et al., 2017; Oliveira et al., 2015). On the other hand, the ability of this bacteria to adapt to different environments results mainly from their high genomic plasticity that is acquired through point mutations, rearrangements, and transfer of genes. The transfer of horizontal gene is the main mechanism for the formation of genomic islands (Barbosa et al., 2014; Dobrindt and Hacker, 2001). Besides that, the genomes of the leptospires differ from the genomes of the other prokaryotes, for example the rRNA genes are not organized in operons, as well as the genomic islands may not have been fully elucidated.

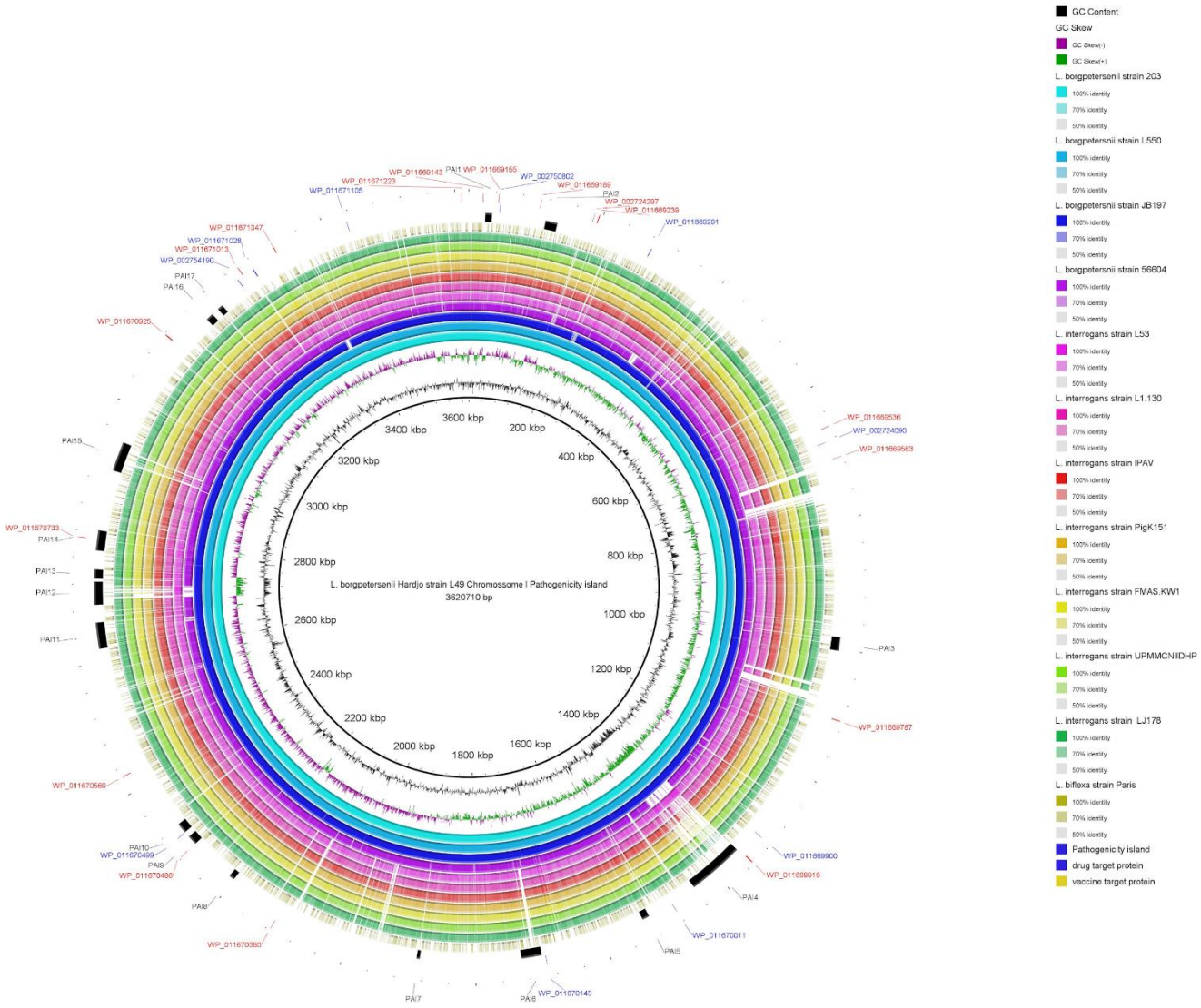

Figure 4. Genomic plasticity analysis with pathogenicity islands predicted in chromosome I using thirteen strains of *L. interrogans* and *L. borgpetersenii* with *L. borgpetersenii* serovar Hardjo strain L49 as reference

Although none of the drug targets are present on a pathogenic island, four drug targets and eight vaccine targets have a score for virulence factor according to the VFDB database (Supplementary Table 2), which indicates that these proteins, with a match in this database, still are good candidates. Since the leptospires genome has some unique characteristics compared to other prokaryotic genomes (Ren et al., 2003), we suggest that the prediction of genomic islands can also be more complex to evaluate using the default of standard Gipsy

software.

Regarding for chromosome II, there is no extraordinary similarity between genomes, and none of the vaccine and drug targets are present, probably due to their smaller size. Therefore, the figure in the BRIG was not performed.

3.3 Subtractive genomics for identification and localization of *L. interrogans* and *L. borgpetersenii* conserved proteins

Using the software OrthoFinder, we found initially for *L. interrogans* 2942 and 3051 genes belonging to the core of isolates from human and animal hosts, respectively; for *L. borgpetersenii* we found 2638 and 2483 genes belonging to the core of isolates from human and animal hosts, respectively. After the genomic subtractive analysis with these core genes, the number was reduced from 843 genes non-homologous to the human host and 1387 genes non-homologous to animal host for *L. interrogans;* the same for *L. borgpetersenii*, 751 genes non-homologous to the human host and 1284 genes non-homologous to the animal host. After the genomic subtractive analysis with these all core genes, the number was reduced to 553 and 533 for *L. interrogans* and *L. borgpetersenii*, respectively. In the final OrthoFinder step, was detected only 221 potential targets for both *L. interrogans* and *L. borgpetersenii* (Figure 1).

As previously described, the final 221 protein presents in the core genome were analyzed for the prediction and subcellular localization by the software SurfG. From these targets, 110 proteins were predicted to be cytoplasmic, and 111 proteins identified as PSE, secreted or membrane (Table 1). Cytoplasmic proteins were selected for the drug discovery stage as they are easier to model molecules in docking, and the other proteins were designed for analysis of vaccine targets.

Table 1. Subcellular localization of target proteins

Localization	Number of proteins
cytoplasmic protein	110
membrane protein	47
PSE	56
secreted protein	8
Total	221

3.4 Vaccine target discovery

Of all proteins predicted as PSE, membrane, or secreted, 17 proteins were classified as good candidate vaccine targets and identified using Vaxign software according to structure, its adhesion capacity to MHC-I and MHC-II and epitope densities. Among the potential vaccine targets, 10 were uncharacterized proteins (five PSE and five membrane proteins), two lipoproteins (PSE), two Ig domain protein IPT/TIG domain protein (PSE), one hydroxyneurosporene synthase CrtC (PSE), one flagellar motor protein MotB (PSE), and one PF07603 family protein (PSE) (Table 2).

Table 2. Functional annotation for vaccine target candidates.

Protein ID	Protein name	location SurfG	adhesin probability	SignalP	TMHMM	Gene UniProt	mass (Da)	length (AA)	predicted epitope	B-cell epitope density
WP_011669916*	Uncharacterized protein	PSE	0.783	yes (14 and15)	0	B9T54_06785	51 008	474	14	0.029
WP_011669787*	Conserved hypothetical lipoprotein	PSE	0.878	yes (17 and 18)	0	LBJ_2078	37 560	365	10	0.027
WP_011670733*	Ig domain protein	PSE	0.812	yes (17 and 18)	0	LEP1GSC123_2800	21 240	207	5	0.024
WP_011669143	Lipoprotein LipL21	PSE	0.652	yes (17 and 18)	0	lipL21	19,774	187	4	0.021
WP_011669239	Hydroxyneurosporene synthase CrtC	PSE	0.671	yes (33 and 34)	1	crtC	42 950	370	8	0.021
WP_011670560*	Uncharacterized protein	PSE	0.550	-	1	LBJ_0937	28 239	247	4	0.016
WP_011669189	Uncharacterized protein	MEMBRANE	0.711	yes (21 and 22)	1	LBJ_2976	14 353	124	2	0.016
WP_011671223	IPT/TIG domain protein	PSE	0.848	-	0	LBJ_0064	29 005	264	4	0.015
WP_011670925*	PF07603 family protein	PSE	0.877	yes (19 and 20)	0	LEP1GSC016_0022	51 620	472	7	0.015
WP_011671013*	flagellar motor protein MotB	PSE	0.514	-	1	motB-1	30 317	275	4	0.014
WP_011670380*	Uncharacterized protein	PSE	0.555	-	2	B9T54_10475	16 864	158	2	0.012
WP_011671047	Uncharacterized protein	PSE	0.779	-	1	LBJ_0292	36 980	321	4	0.012
WP_011669563	Uncharacterized protein	PSE	0.661	-	2	LBJ_2454	13 284	114	1	0.008
WP_011669536*	Uncharacterized protein	MEMBRANE	0.599	-	5	LBJ_2480	22 085	199	1	0.005
WP_011669155	Uncharacterized protein	MEMBRANE	0.550	-	4	B9T54_00165	20 561	175	0	0
WP_002724297	Uncharacterized protein	MEMBRANE	0.519	-	4	LBJ_2910	15 935	140	0	0
WP_011670486	Uncharacterized protein	MEMBRANE	0.550	-	4	LBJ_1759	12 296	109	0	0

***** Proteins with match in VFDB

Table two shows the main characteristics of predicted vaccine targets. Four proteins showed the best MHC adhesion capacity, with index >0.8. According to the number of TMHMM identified it means helices cross the outer membrane several times, forming loops where the epitopes are exposed, allowing better contact with the immune system. Among the 17 proteins five proteins presented one domain, two presented two domains, three presented four domains, and one presented five domains and six had no predicted transmembrane domains. Table 2 also shows the number of predicted peptides with the ability to induce the humoral immune response for each candidate protein for vaccine targets. We found 14 epitopes on the WP_011669916 protein and 10 epitopes on the WP_011669787 protein, which were the ones

with the highest density.

The search in the virulence database (VFDB) resulted in eight proteins with match (marked with an * in table 2). Even though the role of a protein in a pathogen virulence is an important factor, it was not considered as an exclusion factor in the vaccine targets prediction because adhesion and epitope density are characteristics more relevant for classification a candidate as a good one.

The conserved hypothetical lipoprotein (WP_011669787), lipoprotein LipL21 (WP_011669143) and flagellar motor protein MotB (WP_011671013) can be important vaccine targets, whereas since the first *Leptospira* spp genome sequencing both lipoproteins and the motility-linked protein have been related to virulence and pathogenicity of leptospires (Chou et al., 2012; Picardeau et al., 2008; Ren et al., 2003). Another potential vaccine target is hydroxyneurosporene synthase - CrtC (WP_011669239. This enzyme is involved in the biosynthesis of carotenoids spheroidene and spirilloxanthin and catalyzes the hydration of neurosporene to the corresponding hydroxylated carotenoids 1-HO-neurosporene and that of lycopene to 1-HO-lycopene (Steiger et al., 2003). The Ig domain protein (WP_011670733) and IPT/TIG domain (WP_011671223) have an immunoglobulin-like fold. These domains are present in cell surface receptors, such as Met and Ron, as well as in intracellular transcription factors, whose function is related to DNA binding. Noteworthy, the motility and invasion of extracellular matrices are highlight functional features related to virulence and intrinsic at Ron tyrosine kinase receptor and other members of this subfamily (Collesi et al., 1996).

On the other hand, among the vaccine targets, 10 were defined as uncharacterized or hypothetical proteins. Although the factors involved in the virulence are still unclear, some studies have identified hypothetical membrane proteins capable of binding to extracellular matrix components, assisting in the attachment of leptospires to host tissues (Vieira et al., 2014).

The vaccines currently available are inactivated whole-cell products considered to be poorly immunogenic and without contributing to cross-protection against several leptospiral serogroups (Adler, 2015). Vaccination of cattle and dogs is common worldwide, and these should include locally isolated strains of leptospires whenever possible. At the same time, this approach requires continuous surveillance, first to identify the predominant serovars in the region and then to detect the occurrence of new serovars (Ellis, 2015). Besides that, there are still doubts if the use of these vaccines really protects against renal colonization, bacterial shedding, and reproductive failure (Techawiwattanaboon et al., 2019). Thus, several researchers began to seek new ideas for the identification of protein targets and recombinant vaccines obtaining variable results (Evangelista et al., 2017; Garba et al., 2018; Hartwig et al., 2013; Lucas et al., 2011; Teixeira et al., 2018).

Apparently, for the genus *Leptospira*, the concept of a universal vaccine capable of protecting against all pathogenic serovars seems to be still so far away. Meanwhile, the advent of high-throughput sequencing technology has provided an opportunity for the emergence of genome projects which facilitate the process for identifying vaccine candidates directly from the genome sequence (Rappuoli, 2000). Reverse vaccinology makes it possible to search for proteins mainly related to the surface, as these are easily recognized by the host's immune system, combating the microorganism and preventing severe disease.

In the present study, 17 proteins were found capable of adhering to MHC-I and MHC-II with an index greater than 0.51, in other words, capable of inducing cellular or humoral adaptive

immune responses (Solanki and Tiwari, 2018). The subcellular localization of these proteins is in the membrane or the PSE; therefore, the possibility of coming into immediate contact with the host's immune system is elevated. Also, these targets should play an essential role during pathogenesis to increase vaccine effectivenes (Finco and Rappuoli, 2014). Of the 17 vaccine target candidates, two are lipoproteins and spirochete lipoproteins be antigens capable of inducing a protective immune response, confirming their importance in the pathogenesis of leptospirosis. For leptospires, lipoproteins are considered the most prominent proteins present in the cell membrane (Haake, 2000). As previously stated, 10 proteins have been identified as hypothetical or uncharacterized, but these proteins may have membrane adhesion function, and so, blocking the adhesion of leptospires is thought to impair their virulence (Murray et al., 2013).

B-cell epitopes were predicted from the aa sequences of these proteins. These epitopes can be recognized by the immune system and are essential for the development of immunity through antibody production (Vita et al., 2019). In the present study, 14 proteins presented regions with B-cell interaction capacity. Table 2 shows which of the proteins has the highest epitope density. Besides that, of these, only 8 proteins showed transmembrane domains. These helices have regions that cross the outer membrane, forming loops that can better expose the epitopes and allowing straightforward contact with the immune system. However, proteins with many transmembrane domains in their structure should be avoided as it makes purification difficult in trials for vaccine production (Meunier et al., 2016; Rappuoli, 2000).

Considering the characteristics of adhesin probability, importance in the pathogenesis and epitope density, the first five (WP_011669916, WP_011669787, WP_011670733, WP_011669143, WP_011669239) targets presented in the table 2 are good candidates and can be quickly tested in new vaccine formulations and further tested *in vivo*, representing a breakthrough in the prevention and control of leptospirosis.

3.5 Drug target discovery and druggability analysis

The most important analyzes for drug target discovery are based on the selection of predicted proteins as cytoplasmic, considered essential for bacterial survival and importance in the pathogenesis. All 110 cytoplasmic proteins were submitted to MHOLline software for a 3D model prediction. According to identity with the model used in predicting the candidate protein structure, 15 proteins were classified between very high and medium good, and only 10 of them were considered essential/vital for leptospires viability (Supplementary Table 3).

Several factors guide which is the best therapeutic targets. In the last step of the docking, analyzes were evaluated the druggability using DoGSiteScorer and SAVES v5.0 server for essential non-host conserved targets from *L. interrogans* and *L. borgpetersenii*, where can predict at least one druggable cavity for each target. The cavity of each protein exhibiting the highest druggability score (>0.8) and confirmed by Ramachandran plot with residues in most favored regions (> 80%) was subjected to docking analyzes. The Supplementary Table 4 and Supplementary Figure 1 show the druggable pocket with their residues of each target protein. Thus, we examine eight potential targets for the docking studies, only those were considered essential to the pathogen, and at the same time, non-host homology proteins.

Additionally, these eight proteins were submitted to the VFDB, resulting in four best matches (WP_011671028; WP_011670145; WP_002754190; WP_011671105). However, considering the other characteristics of a good candidate for a therapeutic target, all eight proteins followed to the final flexible docking.

Table 3 describes the characteristics of each of the four proteins identified as potential drug candidates and the biological process in which they are involved. According to the structural quality, the protein WP_011671028 (cell division protein FtsZ) was the one with a higher rating. FtsZ is the first protein to move to the division site, and participate in the recruitment of other proteins responsible for the production of a new septum between the cells wall. It forms a contractile ring structure (Z ring) whose function is to regulate the timing and the location of cell division. Besides that, binds GTP and shows GTPase activity (Goehring and Beckwith, 2005). Consequently, there are reports of several studies with compounds that suppress bacterial cell division by inhibiting the FtsZ target because it is an essential bacterial gene (Bi et al., 2019; Haranahalli et al., 2017; Hurley et al., 2016). These data indicate a promising possibility for the development of new bactericidal agents targeting FtsZ.

The protein Imidazoleglycerol-Phosphate Dehydratase seems to be a very promising target, as it has already been studied in the microorganism *Mycobacterium tuberculosis* that causes tuberculosis (Podshivalov et al., 2018; Agapova et al., 2019). It is an enzyme whose function is to catalyze the synthesis of histidine, an essential amino acid for bacterial growth and survival (Lunardi et al., 2013). This enzyme is in the top 50 drug targets design for tuberculosis. Besides, it is also studied as a potential target for new herbicides since it is a protein conserved mainly in microorganisms/plants and absent in mammals (Glynn et al., 2005). Another protein with the potential to target drugs is a SsrA-binding protein, which is very useful for biological functions in all bacteria. SsrA is an all-purpose RNA molecule that functions as both a tRNA and an mRNA. SsrA has the dual function to rescues ribosomes stalled on broken mRNAs and targeting inadequately synthesized protein fragments for degradation. In consequence of its importance in the biology of prokaryotes, already exists a study where this protein has been characterized as a good drug target for diphtheria infection caused by *Corynebacterium diphtheriae* (Jamal et al., 2017).

There are six proteins derived from the Che genes (cheA, cheB, cheR, cheW, cheY and cheZ) involved in the flagellar system and responsible for the rotation and signal transduction pathways (Zhao et al., 2007). The purine-binding chemotaxis protein (CheW) has its function related to chemotaxis and signal transduction and can be considered of great importance for leptospires, whose motility is one of the main virulence factors. Some studies have demonstrated that the inactivation of genes encoding a flagellar motor switch protein damage bacteria mobility and virulence (Li et al., 2010; Liao et al., 2009). Therefore, this is an excellent candidate for targeting a new drug because when it is blocked it reduces the motility and consequently the virulence of leptospires.

Acetolactate synthase is the first common enzyme in the branched-chain amino acid biosynthesis pathway, this essential protein is present in microorganisms and plants but is not found in animals. This quality making it an attractive target for both drug and herbicide discovery. In relation to herbicide discovery, there are already four commercial classes of herbicides that blocking this enzyme including sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl thiobenzoates (Saari; Maxwell, 1997). However, despite the central importance of this enzyme in the metabolism of microorganisms, no inhibitor has been commercialized in antimicrobial agents. Thus, studies indicate that this is also a potential protein for drug targets (Pue; Guddat, 2014).

Table 3. Functional annotation for best potentials drug targets

Protein ID	Protein name	Length aa	Mass (Da)	Gene UniProt	Biological process	Structural quality MHOLline
WP_011671028*	cell division protein FtsZ	401	42 937	ftsZ	division septum assembly/ FtsZ-dependent cytokinesis /protein polymerization	good
WP_011670145*	purine-binding chemotaxis protein CheW	170	19 143	cheW	chemotaxis /signal transduction	medium to good
WP_011671105*	TIGR00730 family Rossman fold protein	185	20 422	LEP1GSC123_4752	cytokinin biosynthetic process	medium to good
WP_002754190*	3-methyl-2-oxobutanoate hydroxymethyltransferase	265	28 806	panB	pantothenate biosynthetic process	medium to good
WP_011669291	Uroporphyrinogen-III C-methyltransferase	277	30 266	cobA	oxidation-reduction process/ siroheme biosynthetic process	medium to good
WP_002750802	Imidazoleglycerol-phosphate dehydratase	193	21 718	hisB	histidine biosynthetic process	medium to good
WP_011670499	SsrA-binding protein	160	18 329	smpB	trans-translation	medium to good
WP_011669900	Acetolactate synthase small subunit	161	17 911	ilvN	branched-chain amino acid biosynthetic process	medium to good

* Proteins with match in VFDB

On the other hand, the 3-methyl-2-oxobutanoate hydroxymethyltransferase protein participates in chemical reactions and pathways resulting in the formation of pantothenate, a B complex vitamin that is a constituent of coenzyme A (CoA). CoA is an essential coenzyme involved in the metabolism of carbohydrates, fats, and proteins in all known living organisms (Begley et al., 2001). Therefore, as its biosynthetic pathway is connected to the primary metabolites, the use of a drug that inhibits 3-methyl-2-oxobutanoate hydroxymethyltransferase will impair this pathway and the final production of pantothenic acid (vitamin B5) and coenzyme A (CoA).

The protein WP_011671105 has not been fully characterized but has a high similarity (Uniprot) to cytokinin (growth promoter of plants) and may be related to the cell division and participate in promoting cell division. The presence of free active cytokinin nucleosides in culture media can confirm this hypothesis (Greene, 1980). Nevertheless, as its real function is not clear in bacteria, it may not be a good candidate for a target like the other proteins.

When we investigated these potential drug-targets proteins in the Drugbank database we found four matches. For cell division protein FtsZ there are three compounds in experimental test (5'-Guanosine-Diphosphate-Monothiophosphate; Guanosine-5'-Diphosphate;

Phosphomethylphosphonic Acid Guanylate Ester) and one approved (citric acid). For 3-methyl-2-oxobutanoate hydroxymethyltransferase there are two compounds in experimental test (2-Dehydropantoate; alpha-Ketoisovalerate). For uroporphyrinogen-III C-methyltransferase there is one drug in experimental test (S-adenosyl-L-homocysteine). For Acetolactate synthase protein there are two drugs in the experimental test (Triethylene glycol and 2-{(9as)-9a-[(1s)-1-Hydroxyethyl]-2,7-Dimethyl-9a,10-Dihydro-5h-Pyrimido[4,5-D][1,3]Thiazolo[3,2-a]Pyrimidin-8-Yl}Ethyl Trihydrogen Diphosphate) and one approved/experimental (Cocarboxylase). This fact helps to proves that these proteins are potential for a drug target, mainly the FtsZ protein.

3.6 Molecular docking and virtual screening

The ligand library was created from 5008 natural compounds obtained from the ZINC database. The choice of compounds to be screened in the docking was due to the role of natural products in the field of pharmacology and drug discovery. This category of molecular compounds is a new resource for pharmaceutical companies working on the development of new drugs (Calixto, 2019). The natural products present advantages in originality in chemical structures compared to other types of synthetic chemical collection, and mainly, they can be easy absorbed and metabolized in the body even presenting complex two-dimensional and three-dimensional structures (Strohl, 2000).

The top 10 compounds were filtered by the AutoDock Vina binding affinity score (Supplementary Table 5), subsequently from the most druggable cavity residues identified in DoGSiteScorer and confirmed by Ramachandran plot (Supplementary Figure 1), for the final flexible docking analysis was performed in the Chimera software (Table 4). As a result, the table shows the predicted protein-ligand interactions for the best compound with each target, contains the compound ID from the ZINC database, the binding affinity of AutoDock Vina, and also the interactions of hydrogen bonds with residues of targets.

Table 4. Molecular docking of drug-like compounds with four drug target proteins according to binding scores/affinity, number of hydrogen bonds, and the residues of proteins interacting with the respective compounds.

Protein ID	ZINC compound ID	Binding affinity score	no. of H-bond	Actives residues
WP_011671028	ZINC04259719	-9	4	PHE317/ ASP244
WP_011670145	ZINC04237088	-8,2	1	GLU116
WP_002754190	ZINC04259385	-8	2	LYS69/ ASN218
WP_011670499	ZINC15709489	-8,1	2	ASP51/ LYS17
WP_011669291	ZINC12605015	-7,8	4	ASP154/ GLY105/ ARG106
WP_011671105	ZINC20503524	-7,1	3	GLY120/ THR 121/ ASP62
WP_002750802	ZINC31154666	-7,4	4	GLU131/ TYR127/ TYR127/ GLN149
WP_011669900	ZINC04236001	-6,8	1	SER125

Finally, docking analyzes was to test the eight drug targets for their effectiveness in binding to the natural compounds obtained from the ZINC database and which bind to the target protein inhibiting its function which is essential to the microorganism. The lower the energy levels (affinity score) and the more hydrogen binding to the active residues, the greater the protein-ligand interaction capacity (Thomsen and Christensen, 2006). Eighty ligands (10 for each target) with high druggability capacity were found and, for each protein target, only one ligand was selected to verify the 3D structural interaction (ZINC04237088, ZINC04259719,

ZINC20503524, ZINC04259385, ZINC15709489, ZINC12605015, ZINC31154666, ZINC04236001) (Supplementary Table 6).

Table 4 and figures 5-6 highlight the target proteins with the respective best natural compound (ligand), with presentation of the binding affinity score, amount of hydrogen bond, and the active residues where this bond occurred. Of all molecular docking (protein-ligand), the compound ZINC04259719 is the molecule with the best affinity score and binds to cell division protein FtsZ (WP_011671028), a target which essential function of recruiting other proteins for produce a new cell wall will be inhibited, harming the multiplication of leptospires. In other words, the target protein WP_011671028 was considered with the best structure quality by MHOLline software and essential for bacterial growth and metabolism. Besides that, some research groups already are studying this protein as a candidate for new drugs target in other bacteria (Bi et al., 2019; Haranahalli et al., 2017; Hurley et al., 2016). Also, ZINC04259719 is identified as the best drug candidate in our analysis, interesting, because it presents in the sulfa group formulation that participates in sulfonamide composition, widely used in human and animal therapy (Guimarães et al., 2010).

Nevertheless, we can observe that despite a higher binding affinity score, the proteins WP_011669291, WP_011671105, and WP_002750802 have three or four hydrogen bonds, which also contributes to a good stability between target and ligand. Thus, using strategies of virtual screening, the compounds ZINC12605015, ZINC20503524, and ZINC31154666 can too be considered good therapeutic molecules to be tested *in vitro* and *in vivo*.

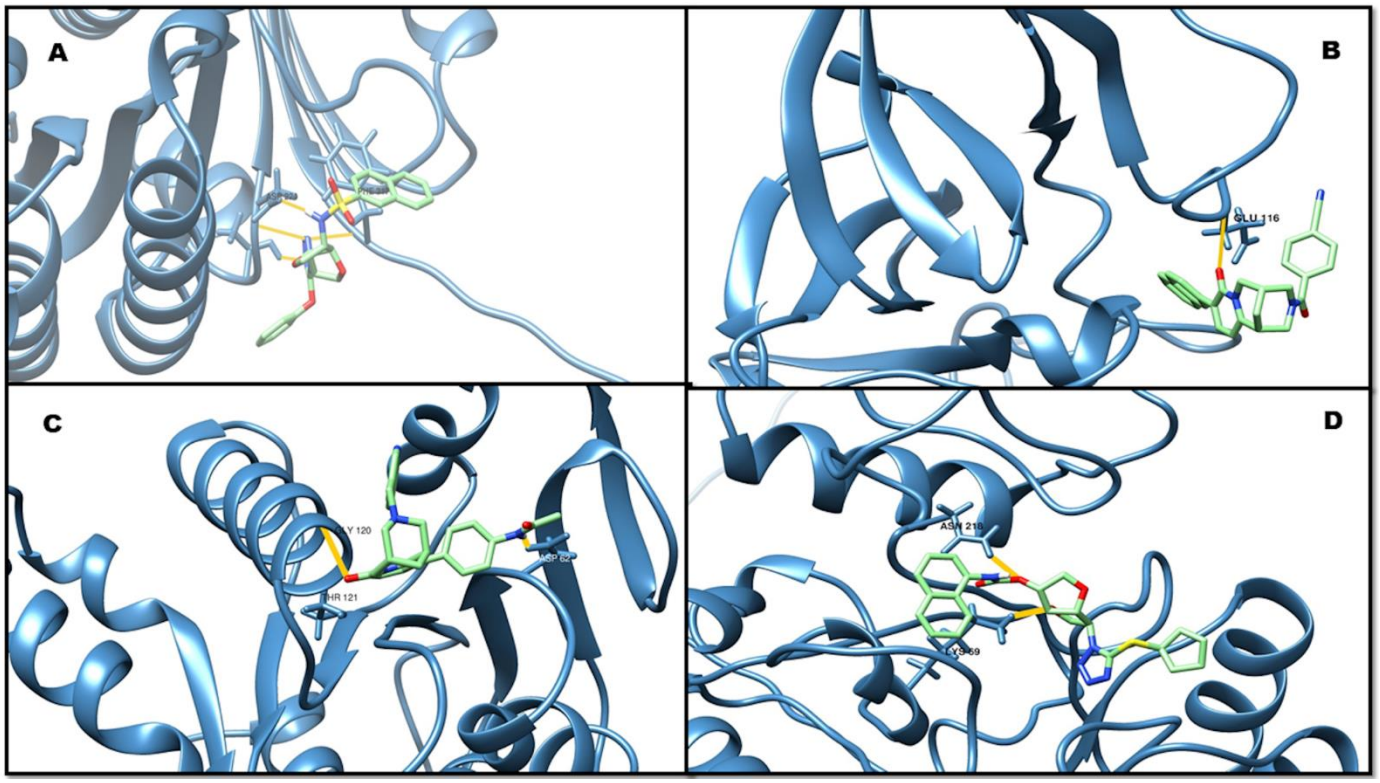

Figure 5. (A) The 3D representation docking analyses of cell division protein FtsZ (WP_011671028) with compound ZINC04259719. (B) The 3D representation docking analyses of purine-binding chemotaxis protein CheW (WP_011670145) with compound ZINC04237088. (C) The 3D representation docking analyses of TIGR00730 family Rossman fold protein (WP_011671105) with compound ZINC20503524. (D) The 3D representation docking analyses of 3-methyl-2-oxobutanoate hydroxymethyltransferase (WP_002754190) with compound ZINC04259385.

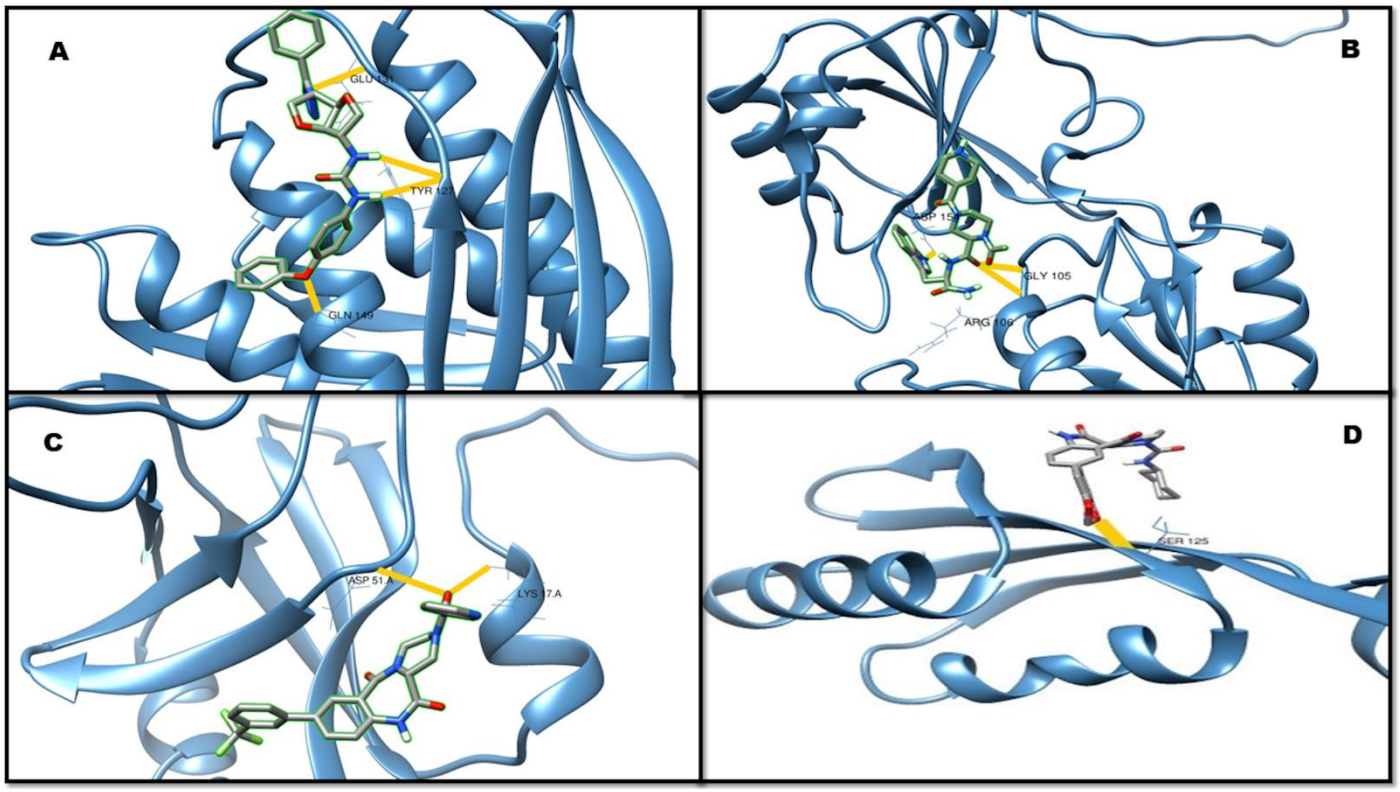

Figure 6. (A) The 3D representation docking analyses of Imidazoleglycerol-phosphate dehydratase (WP_002750802) with compound ZINC31154666. (B) The 3D representation docking analyses of Uroporphyrinogen-III C-methyltransferase (WP_011669291) with compound ZINC12605015. (C) The 3D representation docking analyses of SsrA-binding protein (WP_011670499) with compound ZINC15709489. (D) The 3D representation docking analyses of Acetolactate synthase (WP_011669900) with compound ZINC04236001.

4 CONCLUSION

Leptospirosis is a zoonosis with serious economic impact worldwide, a problem that can be avoided with the development of prophylactic methods and more efficient treatments. In this study, we utilized a bioinformatics pipeline for determining the conserved genes of 21 strains of *L. interrogans* and *L. borgpetersenii*, and afterward evaluated the structure protein 3D to select the best potential vaccine and drug targets. After a careful subtractive genomic analysis, we suggest that eight protein drug targets and 17 protein vaccine targets non -host homologs could be considered for antimicrobial chemotherapy and new vaccine for leptospirosis. The compound ZINC04259719 was the molecule with the best affinity score and binds to cell division protein FtsZ with less energy and more hydrogen bonds. These targets were compared with different databases through molecular docking and should be submitted to future analysis as potential therapeutic resources. The data herein, highlights the great relevance of this study for public and animal health, and can effectively contribute in guiding further research for antibiotics and vaccines development for leptospirosis. In addition, further studies should be conducted with other species of leptospires according to the availability of more complete genomes to obtain more wide vaccine targets.

5 CONFLICT OF INTEREST

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

6 AUTHOR CONTRIBUTIONS

RTC, UPP, and JCF conceived the study. RTC, ST, DDG, UPP, SCS and VACA performed all research. RTC, ST, LMF performed bioinformatics analyzes under supervision of UPP, SCS and VACA. RTC and LMF led the writing and produced the figures. All authors contributed discussing and writing the manuscript. All authors read and approved the final manuscript.

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REFERENCE

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

Adler B. Vaccines against leptospirosis. (2015). *Current topics in microbiology and immunology*, 387, 251–272.

Agapova, Y.K., Timofeev, V.I. & Komolov, A.S. (2019). Molecular Dynamics Study of Triazole Derivative Binding to the Active Site of Imidazole Glycerol Phosphate Dehydratase from Mycobacterium tuberculosis. *Crystallogr. Rep*. 64, 608–610. doi: 10.1134/S1063774519040023

Ågren, J., Sundström, A., Håfström, T., and Segerman, B. (2012). Gegenees: Fragmented alignment of multiple genomes for determining phylogenomic distances and genetic signatures unique for specified target groups. *PLoS One* 7. doi:10.1371/journal.pone.0039107.

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. *BMC Genomics* 12. doi:10.1186/1471-2164-12-402.

Barbosa, E., Röttger, R., Hauschild, A. C., Azevedo, V., and Baumbach, J. (2014). On the limits of computational functional genomics for bacterial lifestyle prediction. *Brief. Funct. Genomics* 13, 398–408. doi:10.1093/bfgp/elu014.

Barinov, A., Loux, V., Hammani, A., Nicolas, P., Langella, P., Ehrlichh, D., et al. (2009). Prediction of surface exposed proteins in Streptococcus pyogenes, with a potential application to other Gram-positive bacteria. *Proteomics* 9, 61–73. doi:10.1002/pmic.200800195.

Begley T.P, Kinsland C., And Strauss E. (2001). The biosynthesis of coenzyme A in bacteria. *Vitam. Horm*. 61, 157-171. 10.1016/s0083-6729(01)61005-7

Bi, F., Song, D., Qin, Y., Liu, X., Teng, Y., Zhang, N., et al. (2019). Discovery of 1,3,4-oxadiazol-2-one-containing benzamide derivatives targeting FtsZ as highly potent agents of killing a variety of MDR bacteria strains. *Bioorganic Med. Chem*. 27, 3179–3193. doi:10.1016/j.bmc.2019.06.010.

Calixto, J. B. (2019). The role of natural products in modern drug discovery. *An. Acad. Bras. Cienc*. 91, 1–7. doi:10.1590/0001-3765201920190105.

Capriles, P. V. S. Z., Guimarães, A. C. R., Otto, T. D., Miranda, A. B., Dardenne, L. E., and Degrave, W. M. (2010). Structural modelling and comparative analysis of homologous, analogous and specific proteins from Trypanosoma cruzi versus Homo sapiens: Putative

drug targets for chagas' disease treatment. *BMC Genomics* 11. doi:10.1186/1471-2164-11-610.

Chou, L., Chen, Y., Lu, C., Ko, Y., Tang, C., Pan, M., et al. (2012). Sequence of Leptospira santarosai serovar Shermani genome and prediction of virulence-associated genes. *Gene* 511, 364–370. doi:10.1016/j.gene.2012.09.074.

Collesi, C., Santoro, M. M., Gaudino, G., and Comoglio, P. M. (1996). A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype. *Mol. Cell. Biol*. 16, 5518–5526. doi:10.1128/mcb.16.10.5518.

Conrad, N. L., Cruz, McBride, F. W., Souza, J. D., Silveira, M. M., Félix, S., Mendonça, K. S., et al. (2017). LigB subunit vaccine confers sterile immunity against challenge in the hamster model of leptospirosis. *PLoS Negl. Trop. Dis*. 11, 1–20. doi:10.1371/journal.pntd.0005441.

Costa, J. M., Moura, C. S., Pádua, C. A. M., Vegi, A. S. F., Magalhães, S. M. S., Rodrigues, M. B., et al. (2019). Medida restritiva para comercialização de antimicrobianos no Brasil : resultados alcançados. *Rev. Saude Publica*, 53–68.

Daher, E. F., Silva, G. B., De Abreu, K. L. S., Mota, R. M. S., Batista, D. V., Rocha, N. A., et al. (2012). Leptospirosis-associated acute kidney injury: Penicillin at the late stage is still controversial. *J. Clin. Pharm. Ther*. 37, 420–425. doi:10.1111/j.1365-2710.2011.01312.x.

Dellagostin, A. O., Grassmann AA., Hartwig DD., Felix SR., et al., (2011). Recombinant vaccines against leptospirosis. *Hum. Vaccin*. 7, 1215–1224. doi:10.4161/hv.7.11.17944.

Dellagostin, O. A., Grassmann, A. A., Rizzi, C., Schuch, R. A., Jorge, S., Oliveira, T. L., Hartwig, D. D. (2017). Reverse Vaccinology: An Approach for Identifying Leptospiral Vaccine Candidates. *International journal of molecular sciences*, 18(1), 158. doi:10.3390/ijms18010158

Dobrindt, U., and Hacker, J. (2001). Whole genome plasticity in pathogenic bacteria. *Curr. Opin. Microbiol.* 4, 550–557. doi:10.1016/S1369-5274(00)00250-2.

Ellis, W. A. Animal leptospirosis. (2015). *Curr. Top. Microbiol. Immunol*. 387, 99–137.

Emms, D. M., and Kelly, S. (2019). OrthoFinder: Phylogenetic orthology inference for comparative genomics. *Genome Biol*. 20, 1–14. doi:10.1186/s13059-019-1832-y.

Evangelista, K. V., Lourdault, K., Matsunaga, J., and Haake, D. A. (2017). Immunoprotective properties of recombinant LigA and LigB in a hamster model of acute leptospirosis. *PLoS One* 12, 1–21. doi:10.1371/journal.pone.0180004.

Facimoto, C. T., Chideroli, R. T., Di Santis, G. W., Gonçalves, D. D., do Carmo, A. O., Kalapothakis, E., et al. (2019). Complete genome sequence of Francisella noatunensis subsp. orientalis strain F1 and prediction of vaccine candidates against warm and cold-water fish francisellosis. *Genetics and Molecular Research*, 18, 1-10. doi: 10.4238/gmr18302.

Finco, O., and Rappuoli, R. (2014). Designing vaccines for the twenty-first century society. *Front. Immunol.* 5, 1–6. doi:10.3389/fimmu.2014.00012.

Galardini, M., Biondi, E. G., Bazzicalupo, M., and Mengoni, A. (2011). CONTIGuator: A bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol*. Med. 6, 11. doi:10.1186/1751-0473-6-11.

Garba, B., Bahaman, A. R., Zakaria, Z., Bejo, S. K., Mutalib, A. R., Bande, F., et al. (2018). Antigenic potential of a recombinant polyvalent DNA vaccine against pathogenic leptospiral infection. *Microb. Pathog*. 124, 136–144. doi:10.1016/j.micpath.2018.08.028.

Glynn, S. E., Baker, P. J., Sedelnikova, S. E., Davies, C. L., et al. (2005). Structure and mechanism of imidazoleglycerol-phosphate dehydratase. *Structure*. 13:1809–1817

Goehring, N. W., and Beckwith, J. (2005). Diverse paths to midcell: Assembly of the bacterial cell division machinery. *Curr. Biol*. 15, 514–526. doi:10.1016/j.cub.2005.06.038.

Grassmann, A. A., Souza, J. D., and McBride, A. J. A. (2017). A universal vaccine against leptospirosis: Are we going in the right direction? *Front. Immunol*. 8, 1–8. doi:10.3389/fimmu.2017.00256.

Greene, E. M. Cytokinin Production by Microorganisms: Botanical Review. (1980). *New York Botanical Garden Press*, 46, 25-74.

Guimaraes, D. O., Momesso, L. S., Pupo, M. T. (2010). Antibióticos: importância terapêutica e perspectivas para a descoberta e desenvolvimento de novos agentes. *Quím. Nova*, 33, (3), 667-679. Doi: 10.1590/S0100-40422010000300035.

Haake, D. A. (2000). Spirochaetal lipoproteins and pathogenesis. *Microbiology* 146, 1491–1504. doi:10.1099/00221287-146-7-1491.

Haranahalli, K., Tong, S., Ojima, I., Brook, S., Discovery, D., and Brook, S. (2017). Recent advances in the discovery and development of antibacterial agents targeting the cell-division protein FtsZ Krupanandan. *HHS Public Access* 24, 6354–6369. doi:10.1016/j.bmc.2016.05.003.Recent.

Hartwig, D. D., Forster, K. M., Oliveira, T. L., Amaral, M., McBride, A. J. A., and Dellagostina, O. A. (2013). A prime-boost strategy using the novel vaccine candidate, LemA, protects hamsters against leptospirosis. *Clin. Vaccine Immunol*. 20, 747–752. doi:10.1128/CVI.00034-13.

He, Y., Xiang, Z., and Mobley, H. L. T. (2010). Vaxign: The first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J. Biomed. Biotechnol.* 2010. doi:10.1155/2010/297505.

Hurley, K. A., Santos, T. M. A., Nepomuceno, G. M., Huynh, V., Shaw, J. T., and Weibel, D. B. (2016). Targeting the Bacterial Division Protein FtsZ. *J. Med. Chem.* 59, 6975–6998. doi:10.1021/acs.jmedchem.5b01098.

Huson, D. H., and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23, 254–267. doi:10.1093/molbev/msj030.

Irwin, J. J., and Shoichet, B. K. (2005). ZINC - A free database of commercially available compounds for virtual screening. *J. Chem. Inf. Model.* 45, 177–182. doi:10.1021/ci049714+.

Jamal, S. B., Hassan, S. S., Tiwari, S., Viana, M. V., De Jesus Benevides, L., Ullah, A., et al. (2017). An integrative in-silico approach for therapeutic target identification in the human pathogen Corynebacterium diphtheriae. *PLoS One* 12, 1–25. doi:10.1371/journal.pone.0186401.

Kraemer, S. A., Ramachandran, A., and Perron, G. G. (2019). Antibiotic pollution in the environment: From microbial ecology to public policy. *Microorganisms* 7, 1–24. doi:10.3390/microorganisms7060180.

Krogh, A., Larsson, B., Von Heijne, G., and Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* 305, 567–580. doi:10.1006/jmbi.2000.4315.

Lee, H. S., Guptill, L., Johnson, A. J., and Moore, G. E. (2014). Signalment changes in canine leptospirosis between 1970 and 2009. *J. Vet. Intern. Med.* 28, 294–299. doi:10.1111/jvim.12273.

Li, C., Xu, H., Zhang, K., and Liang, F. T. (2010). Inactivation of a putative flagellar motor switch protein FliG1 prevents Borrelia burgdorferi from swimming in highly viscous media and blocks its infectivity. *Mol. Microbiol.* 75, 1563–1576. doi:10.1111/j.1365-2958.2010.07078.x.

Liao, S., Sun, A., Ojcius, D. M., Wu, S., Zhao, J., and Yan, J. (2009). Inactivation of the fliY gene encoding a flagellar motor switch protein attenuates mobility and virulence of Leptospira interrogans strain Lai. *BMC Microbiol.* 9. doi:10.1186/1471-2180-9-253.

Liu, B., Zheng, D., Jin, Q., Chen, L., and Yang, J. (2019). VFDB 2019: A comparative

pathogenomic platform with an interactive web interface. *Nucleic Acids Res.* 47, D687–D692. doi:10.1093/nar/gky1080.

Lucas, D. S., Cullen, P. A., Lo, M., Srikram, A., Sermswan, R. W, et al. (2011). Recombinant LipL32 and LigA from Leptospira are unable to stimulate protective immunity against leptospirosis in the hamster model. *Vaccine* 29, 3413–3418. 10.1016/j.vaccine.2011.02.084

Lunardi, J., Nunes, J. E. S., Bizarro, C.V., Basso, L. A, Santos, D. S, Machado, P. (2013). Targetting the histidine pathway in Mycobacterium tuberculosis. *Curr Top Med Chem* 13: 2866– 2884. doi: 10.2174/15680266113136660203.

Martins, G., and Lilenbaum, W. (2013). The panorama of animal leptospirosis in Rio de Janeiro, Brazil, regarding the seroepidemiology of the infection in tropical regions. *BMC Vet. Res.* 9, 237. doi:10.1186/1746-6148-9-237.

Meunier, M., Guyard-Nicodème, M., Hirchaud, E., Parra, A., Chemaly, M., and Dory, D. (2016). Identification of novel vaccine candidates against campylobacter through reverse vaccinology. *J. Immunol. Res.* 2016. doi:10.1155/2016/5715790.

Mondal, S. I., Ferdous, S., Nurnabi, J. A., Akter, A., Mahmud, Z., Islam, M. M., et al. (2015). Identification of potential drug targets by subtractive genome analysis of Escherichia coli O157 : H7 : an in silico approach. *Adv. Appl. Bioinforma. Chem.* 8, 49–63.

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., et al. (2009). AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *J Comput Chem.* 30, 2785–2791. doi:10.1002/jcc.21256.AutoDock4.

Murray, G. L., Lo, M., Bulach, D. M., Srikram, A., Seemann, T., Quinsey, N. S., et al. (2013). Evaluation of 238 antigens of Leptospira borgpetersenii serovar Hardjo for protection against kidney colonisation. *Vaccine* 31, 495–499. doi:10.1016/j.vaccine.2012.11.028.

O'Boyle, N. M., Banck, M., James, C. A., Morley, C., Vandermeersch, T., and Hutchison, G. R. (2011). Open Babel: An Open chemical toolbox. *J. Cheminform.* 3, 33. doi:10.1186/1758-2946-3-33.

Oliveira, T. L., Grassmann, A. A., Schuch, R. A., Neto, A. C. P. S., Mendonça, M., Hartwig, D. D., et al. (2015). Evaluation of the leptospira interrogans outer membrane protein OmpL37 as a vaccine candidate. *PLoS One* 10, 1–13. doi:10.1371/journal.pone.0142821.

Podshivalov, D. D., Mandzhieva, Yu. B., Sidorov-Biryukov, D. D., et al. (2018). Discovery of Selective Inhibitors of Imidazoleglycerol-Phosphate Dehydratase from *Mycobacterium tuberculosis* by Virtual Screening. *Crystallogr. Rep.* 63 (1), 74. doi: 10.1134/S1063774518010133

Paes, A. C. Leptospirose canina. (2016). In: Megid, J., Ribeiro, M. G, Paes, A. C. *Doenças Infecciosas em Animais de Produção e de Companhia***.** Rio de Janeiro: Editora Roca, 1ed, p. 356-377.

Petersen, T. N., Brunak, S., Von Heijne, G., Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods*, 2011;8: 785–786. 10.1038/nmeth.1701

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., et al. (2004). UCSF Chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612. doi:10.1002/jcc.20084.

Picardeau, M., Bulach, D. M., Bouchier, C., Zuerner, R. L., Zidane, N., Wilson, P. J., et al. (2008). Genome Sequence of the Saprophyte Leptospira biflexa Provides Insights into the Evolution of Leptospira and the Pathogenesis of Leptospirosis. *PLoS ONE* 3, 1–9. doi:10.1371/journal.pone.0001607.

Pinto, P. S., Libonati, H., and Lilenbaum, W. (2017). A systematic review of leptospirosis on dogs, pigs, and horses in Latin America. *Trop. Anim. Health Prod.* 49, 231–238. doi:10.1007/s11250-016-1201-8.

Pue, N., Guddat, L. W. (2014). Acetohydroxyacid synthase: a target for antimicrobial drug discovery. *Curr Pharm Des*. 20(5), 740–753. doi:10.2174/13816128113199990009

Rappuoli, R. (2000). Reverse vaccinology Rino Rappuoli. *Curr. Opin. Microbiol.*, 445–450.

Rappuoli R., Bottomley MJ., D'oro U., Finco O., De Gregorio E. (2016). Reverse vaccinology 2.0: Human immunology instructs vaccine antigen design. *J Exp Med*, 213, 469–481. doi:10.1084/jem.20151960

Ratet, G., Veyrier, F. J., Fanton d'Andon, M., Kammerscheit, X., Nicola, M. A., Picardeau, M., et al. (2014). Live Imaging of Bioluminescent Leptospira interrogans in Mice Reveals Renal Colonization as a Stealth Escape from the Blood Defenses and Antibiotics. *PLoS Negl. Trop. Dis.* 8. doi:10.1371/journal.pntd.0003359.

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

Saari, L. L., Maxwell, C. A. (1997). Target-Site Resistance for Acetolactate Synthase Inhibitor Herbicides. In: De Prado R., Jorrín J., García-Torres L. (eds) Weed and Crop Resistance to Herbicides. Springer, Dordrecht. doi: 10.1007/978-94-011-5538-0_8

Santos, A. R., Pereira, V. B., Barbosa, E., Baumbach, J., Pauling, J., Röttger, R., et al. (2013). Mature Epitope Density - A strategy for target selection based on immunoinformatics and exported prokaryotic proteins. *BMC Genomics* 14, 1–11. doi:10.1186/1471-2164-14-S6-S4.

Soares, S. C., Geyik, H., Ramos, R. T. J., de Sá, P. H. C. G., Barbosa, E. G. V., Baumbach, J., et al. (2016). GIPSy: Genomic island prediction software. *J. Biotechnol.* 232, 2–11. doi:10.1016/j.jbiotec.2015.09.008.

Solanki, V., and Tiwari, V. (2018). Subtractive proteomics to identify novel drug targets and reverse vaccinology for the development of chimeric vaccine against Acinetobacter baumannii. *Sci. Rep.* 8, 1–19. doi:10.1038/s41598-018-26689-7.

Steiger, S., Mazet, A., and Sandmann, G. (2003). Heterologous expression, purification, and enzymatic characterization of the acyclic carotenoid 1,2-hydratase from Rubrivivax gelatinosus. *Arch. Biochem. Biophys.* 414, 51–58. doi:10.1016/S0003-9861(03)00099-7.

Strohl, W. R. (2000). The role of natural products in a modern drug discovery program. *Drug Discov. Today* 5, 39–41. doi:10.1016/S1359-6446(99)01443-9.

Techawiwattanaboon, T., Barnier-Quer, C., Palaga, T., Jacquet, A., Collin, N., Sangjun, N., et al. (2019). Reduced renal colonization and enhanced protection by leptospiral factor h binding proteins as a multisubunit vaccine against leptospirosis in hamsters. *Vaccines* 7. doi:10.3390/vaccines7030095.

Teixeira, A. F., Fernandes, L. G. V., Souza Filho, A., Souza, G. O., Vasconcellos, S. A., Heinemann, M. B., et al. (2018). Evaluation of Lsa46 and Lsa77 Leptospiral Proteins for Their Immunoprotective Activities in Hamster Model of Leptospirosis. *Biomed Res. Int.* 2018, 17–22. doi:10.1155/2018/1813745.

Thomsen, R., and Christensen, M. H. (2006). MolDock: A new technique for high-accuracy molecular docking. *J. Med. Chem.* 49, 3315–3321. doi:10.1021/jm051197e.

Trott, O., and Olson, A. (2010). Autodock vina: improving the speed and accuracy of docking. *J. Comput. Chem.* 31, 455–461. doi:10.1002/jcc.21334.AutoDock.

Uniprot Consortium TU. The universal protein resource (UniProt). *Nucleic Acids Res.* 2008;36 (Database issue):D190–D195. doi:10.1093/nar/gkm895

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

Vincent, A.T.; Schiettekatte, O.; Goarant, C.; Neela, V.K.; Bernet, E.; Thibeaux, R.; Ismail, N.; Mohd Khalid, M.K.N.; Amran, F.; Masuzawa, T.; et al. Revisiting the taxonomy and

evolution of pathogenicity of the genus Leptospira through the prism of genomics. P*LoS Negl. Trop. Dis*. 13, p. e0007270, 2019. https://doi.org/10.1371/journal.pntd.0007270

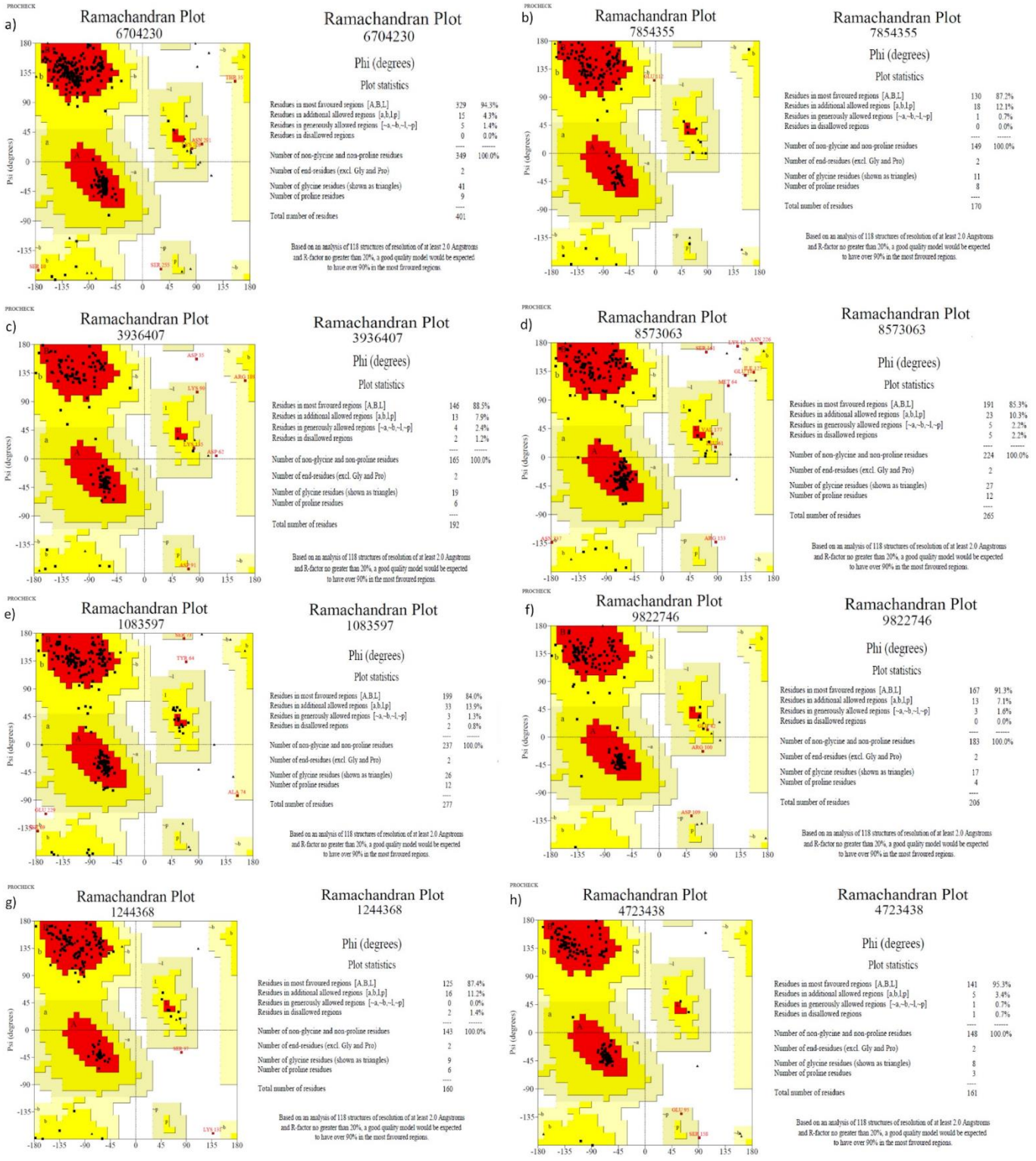
Vita, R., Mahajan, S., Overton, J. A., Dhanda, S. K., Martini, S., Cantrell, J. R., et al. (2019). The Immune Epitope Database (IEDB): 2018 update. *Nucleic Acids Res.* 47, D339–D343. doi:10.1093/nar/gky1006.

Volkamer, A., Kuhn, D., Rippmann, F., and Rarey, M. (2012). Dogsitescorer: A web server for automatic binding site prediction, analysis and druggability assessment. *Bioinformatics* 28, 2074–2075. doi:10.1093/bioinformatics/bts310.

Wishart, D. S., Feunang, Y. D., Guo, A. C., Lo, E. J., Marcu, A., Grant, J. R., et al. (2018). DrugBank 5.0: A major update to the DrugBank database for 2018. *Nucleic Acids Res.* 46, D1074–D1082. doi:10.1093/nar/gkx1037.

Zeng, L. B., Wang, D., Hu, N. Y., Zhu, Q., Chen, K., Dong, K., et al. (2017). A novel pan-genome reverse vaccinology approach employing a negative-selection strategy for screening surface-exposed antigens against leptospirosis. *Front. Microbiol.* 8, 396. doi:10.3389/fmicb.2017.00396.

Zhang, R., Ou, H., and Zhang, C. (2004). DEG: a database of essential genes. *Nucleic Acids Res.* 32, 271D – 272. doi:10.1093/nar/gkh024.



Supplementary Figure 1. Ramachandra plot with residues in most favored regions of four protein candidates drug target. a) WP_011671028, b) WP_011670145, c) WP_011671105, d) WP_002754190, e) WP_011669291, f) WP_002750802, g) WP_011670499, h) WP_011669900

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Supplementary table 1. General information about the genomes of leptospira strains used in this work.

Specie	Serovar	strain	Host	Isolation source	size (MB)	GC (%)	gene number	protein number	Sequencing Technology	GenBank number
Leptospira interrogans	Lai	56601	Homo sapiens	blood	469813	35.1	3741	3683	-	chromosome I: NC_004342.2 Cr I, chromosome II: NC_004343.2
Leptospira interrogans	Hardjo	norma	cattle	urine	476215	35.0	4235	3876	454	chromosome I:NZ_CP012603.1 chromosome II:NZ_CP012604.1
Leptospira interrogans	Bratislava	Pigk151	swine	kidney	472158	35.0	4226	3832	Roche gsFLX Titanium; Illumina; Sanger	chromosome I:NZ_CP011410.1 chromosome II:NZ_CP011411.1
Leptospira interrogans	Hardjo	L53	cattle	urine	474240	35.1	4222	3864	Illumina Miseq	chromosome 1:NZ_CP043041.1 chromosome 2:NZ_CP043039.1 plasmid p1:NZ_CP043

										040.1
Leptospira interrogans	Lai	IPAV		derived by prolonged laboratory passage from a highly virulent ancestral strain isolated in China	470853	35.1	4138	3856	-	chromosome 1:NC_017551.1 chromosome 2:NC_017552.1
Leptospira interrogans	Linhai	56609	Homo sapiens	blood	491565	35.07	4466	4057	454	chromosome 1:NZ_CP006723.1 chromosome 2:NZ_CP006724.1 plasmid lcp1:NZ_CP006725.1

Leptospira interrogans		isolate 1	Homo sapiens	blood	474009	34.99	4116	3709	-	chromosome I:NZ_CP039258.1/CP039258.1 chromosome II:NZ_CP039259.1 plasmid pLiLS1:NZ_CP039260.1
Leptospira interrogans		isolate 2	Homo sapiens	blood	465114	35.0	4044	3703	-	chromosome I:NZ_CP039256.1/CP039256.1 chromosome II:NZ_CP039257.1/CP039257.1
Leptospira interrogans		isolate 3	Homo sapiens	blood	507174	35.18	4539	4116	-	chromosome I:NZ_CP039283.1 chromosome II:NZ_CP039284.1 plasmid pLiSL1:NZ_CP039285.1
Leptospira interrogans	Copenhageni	FDAARGOS_203	Homo sapiens	unknown	463076	35.0	4023	3748	PacBio; Illumina	chromosome:NZ_CP020414.2 plasmid unnamed1:NZ_CP020413.2

Leptospira interrogans	Copenhageni	Fiocruz L1-130	Homo sapiens	unknown	462737	35.0	4026	3734	-	chromosome I:NC_005823.1 chromosome II:NC_005824.1
Leptospira interrogans	Canicola	LJ138	canine	unknown	475849	34.94	4165	3832	-	chromosome 1:NZ_CP044509.1 chromosome 2:NZ_CP044510.1 plasmid p1:NZ_CP044511.1
Leptospira interrogans	Canicola	611	Homo sapiens	unknown	475.534	34.98	4169	3820	-	chromosome 1:NZ_CP044513.1 chromosome 2:NZ_CP044514.1 plasmid p1:NZ_CP044515.1
Leptospira interrogans	Manilae	UP-MMC-NIID LP	*Mus musculus*	kidney	46.674	34.98	4095	3776	-	chromosome 1:NZ_CP011931.1 chromosome 2:NZ_CP011932.1 plasmid

										pLIMLP1:NZ_CP011933.1
Leptospira interrogans	Manilae	UP-MMC-NIID HP	*Mus musculus*	kidney	466.735	34.98	4095	3768	-	chromosome 1:NZ_CP011934.1 chromosome 2:NZ_CP011935.1 plasmid pLIMHP1:NZ_CP011936.1
Leptospira borgpetersenii	Hardjo	L550	*Homo sapiens*	blood	393.178	40.20	3847	3288	-	chromosome 1:NC_008508.1 chromosome 2:NC_008509.1
Leptospira borgpetersenii	Hardjo	JB197	Cattle	Kidney	387.623	40.22	3770	3222	-	chromosome 1:NC_008510.1 chromosome 2:NC_008511.1

Leptospira borgpetersenii	Ceylonica	Piyasena	*Homo sapiens*	blood	399.278	40.12	3792	3460	Illumina; Oxford Nanopore	chromosome 1:NZ_CP026671.1 chromosome 2:NZ_CP026672.1
Leptospira borgpetersenii	Hardjo	L49	Cattle	urine	393.591	40.19	3816	3262	Illumina Miseq	chromosome 1:NZ_CP033440.1 chromosome 2:NZ_CP033441.1
Leptospira borgpetersenii	Hardjo	203	*Homo sapiens*	Kidney	390.733	40.20	3791	3244	PacBio; Illumina	chromosome I:NZ_CP021412.1 chromosome II:NZ_CP021413.1
Leptospira borgpetersenii	Ballum	56604	*Rattus rattus*	Kidney	403.758	40.21	3842	3395	454	chromosome 1:NZ_CP012029.1 chromosome 2:NZ_CP012030.1 plasmid lbp1:NZ_CP012031.

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Supplemantary table 2. Search result in the virulence factor database (VFDB) with the score for each target

Protein ID – Drug target	**Score database VFDB**
WP_011671028 cell division protein FtsZ	33
WP_011669291 uroporphyrinogen-III C-methyltransferase	28
WP_011670145 purine-binding chemotaxis protein CheW	108
WP_002750802 imidazoleglycerol-phosphate dehydratase HisB	No hit found
WP_011671105 MULTISPECIES: TIGR00730 family Rossman fold protein	35
WP_011669900 acetolactate synthase small subunit	28
WP_002754190 3-methyl-2-oxobutanoate hydroxymethyltransferase	30
WP_011670499 SsrA-binding protein	28
Protein ID – Vaccine target	**Score database VFDB**
WP_011669155 hypothetical protein	27
WP_002724297 MULTISPECIES: hypothetical protein	No hit found
WP_011669143 lipoprotein LipL21	27
WP_011670733 hypothetical protein	45
WP_011670380 hypothetical protein	30
WP_011669787 DUF1565 domain-containing protein	33
WP_011669916 hypothetical protein	38
WP_011669239 carotenoid 1,2-hydratase	29
WP_011671223 DNA-binding protein	28
WP_011670925 DUF1566 domain-containing protein	34
WP_011671013 flagellar motor protein MotB	77
WP_011670560 hypothetical protein	34
WP_011671047 hypothetical protein	29
WP_011669189 MULTISPECIES: hypothetical protein	27
WP_011669563 DUF962 domain-containing protein	27
WP_011670486 hypothetical protein	27
WP_011669536 hypothetical protein	31

Supplementary Table 3: Template ID used by MHOLline for Identified targets for modeling with identity.

Protein Name	Template ID	Idenity (%)
WP_011670011	3nrk	>=50% and <75%
WP_011671028	5xdt	>=50%
WP_011669291	1va0	>=35% and <50%
WP_011670145	2qdl	>=35% and <50%
WP_002750802	5ekw	>=35% and <50%
WP_011671105	2a33	>=35% and <50%
WP_011669900	2f1f	>=35% and <50%
WP_002754190	1o66	>=35% and <50%
WP_011670499	1wjx	>=35% and <50%
WP_002724090	1hqk	>=35% and <50%

Supplementary Table 4. Identified druggable pocket with its volume, surface area and drug score of each target protein obtained from DoGSiteScorer.

Protein ID	Volume (Å3)	Surface area (Å2)	Drug score	Residues
WP_011671028.1	1854.41	1998.18	0.81	SER10, SER11, PRO12, ALA13, MET99, PHE101, VAL130, VAL132, ILE152, THR161, LEU162, ILE163, LEU164, ILE165, VAL186, ASP189, ILE190, LEU191, ASN193, ALA194, ARG196, GLY197, ILE198, ASP200, ILE201, ILE202, ASN203, ASN204, PRO205, GLY206, ILE208, ASN209, VAL210, ASP211, PHE212, ASP214, VAL215, MET219, ASP224, ALA225, VAL226, MET227, GLY228, VAL229, GLY230, GLU231, TYR244, ALA245, ILE246, ASN247, ASN248, SER249, LEU250, LEU251, ASP252, SER253, ALA254, SER255, THR257,GLY258, LEU263, ILE264, ASN265, VAL266, SER267, GLY268, GLY297, LEU298, HIS299, ASP301, LEU304, LYS307, ILE308, ARG309, VAL310, THR311, VAL312, ILE313, ALA314, THR315, PHE317, ARG319
WP_011670145.1	401.09	2025.7	0.81	LEU10, THR11, PHE12, CYS13, GLU16, GLU17, CYS18, TYR19, GLY20, ILE21, LEU24, HIS25, ILE26, LYS27, ILE29, PHE44, ILE45, VAL59, LEU61, LYS64, PHE65, LYS75, THR76, CYS77, VAL78, GLU79, VAL81, GLU82, ILE83, ASN88, ASP90, GLN91, LYS92, GLU93, LYS94, THR95, ASP96, LEU97, ILE99, VAL107, ILE108, SER109, ILE110, ASN113, ASP114, ILE115, GLU116, ALA118, PRO119, THR120, GLY122, SER123, LYS126, VAL127, PHE129, ILE130, LEU131, GLY132, MET133, ALA134, LYS135, GLN136, SER138, GLY139, PHE140, ILE141
WP_011671105.1	1483.65	2370.26	0.81	SER21, ARG22, SER23, THR25, ASN26, PRO27, VAL28, TYR29, THR30, GLU31, ALA32, ALA33, ASN44, LEU47, VAL48, GLY50, GLY51, ALA52, CYS54, ILE56, MET57, GLY58, THR59, ILE60, ALA61, ASP62, ALA63, VAL64, MET65, GLU66, LYS67, GLY68, GLY69, GLY70, VAL71, SER72, ILE74, ILE75, ASP77, LEU79, SER80, ILE81, LYS82, GLU83, VAL84, LYS85, HIS86, ARG88, VAL89, LYS90, ASP91, LEU92, MET93, ILE94, MET98, ARG101, LYS102, PHE103, ARG104, MET105, GLU107, LYS108, SER109, SER110, GLY111, PHE112, ILE113, LEU115, GLY117, GLY118, ILE119, GLY120, THR121, GLU124, PRO140, LEU184, LEU187,

				HIS188
WP_002754190.1	2169.02	2787.56	0.81	MET48, PHE50, GLN51, ILE65, TYR66, HIS67, THR68, LYS69, VAL70, VAL71, ARG73, GLY74, PRO76, PHE79, VAL80, ILE81, ALA82, ASP83, PHE86, SER88, TYR89, SER92, ARG102, MET103, MET104, LYS105, GLU106, THR107, LYS129, ILE131, PHE149, GLY151, HIS152, VAL154, GLN155, GLY156, LYS157, LEU182, GLU183, MET184, ILE204, VAL214, LEU215, VAL216, ASN218, ASP219, PHE231, LEU232
WP_011669291	1702.32	2017.03	0.81	ALA20, GLY21, PRO22, GLY23, ASN24, PRO25, LEU28, ASP45, ALA46, LEU47, LEU48, ASP49, PRO50, PHE52, TYR64, VAL65, GLY66, LYS67, ARG68, HIS72, SER73, ALA74, ILE79, ARG95, LYS97, GLY98, GLY99, ASP100, PRO101, VLA103, PHE104, GLY105, ARG106, GLU110, SER128, SER129, LEU130, ALA132, MET153, ASP154, HIS156, THR157, VAL158, SER160, THR163, LEU177, PHE178, MET179, GLY180, THR181, ALA182, LEU184, LEU204, VAL205, GLU206, ASN207, ALA208, SER209, LEU210, ASN214, ILE215, GLN216, GLU229, LYS230, LYS231, THR232, LYS233, GLY234, PRO235, GLY236, ILE237, ILE238
WP_002750802.1	686.27	658.52	0.88	MET29, THR41, GLU42, ILE43, PHE45, PHE46, MET49, LEU50, ILE53, ILE59, LEU61, LEU63, LEU65, CYS73, SER76, VAL77, THR80, MET84, GLY85, ILE88, VAL111, THR113, THR114, VAL117, PHE124, PHE125, TYR127, THR128, GLY129, PRO130, GLU131, LEU132, THR133, TYR139, LEU143, SER144, LEU145, PHE147, LEU148, GLN149, LEU151, ALA152, ALA155, LYS156, MET157, ASN158, LEU159, VAL161, PHE162, VAL163, TYR165, GLY166, ASP167, ASN168, ARG169, VAL172, HIS173, ILE176, PHE177, LEU180, GLY181, LEU184
WP_011670499.1	1521.09	2283.16	0.82	HIS10, SER11, PRO12, LEU13, VAL14, ASN15, LYS16, LYS17, ALA18, ASN21, PHE22, LEU24, VAL25, SER26, PHE27, ILE28, GLU29, ALA30, ILE32, LEU34, LEU42, LYS45, GLY47, ASN48, LEU49, THR50, ASP51, ALA52, PHE53, ALA54, LYS55, ILE56, VAL61, LEU63, GLU64, ASN65, PHE66, ILE68, ASN73, GLY74, ILE82, ARG83, PRO84, ARG85, LEU87, LEU89, GLU93, LYS96, LEU97, GLU98, ARG99, GLN100, VLA101, LYS102, GLU103, GLY105, LEU106, VAL107, LEU108, VAL109, ALA110, THR111, LYS112, VAL113, TYR114, PHE115, VAL121, VAL123, ILE125, ALA126, VAL127, GLY128, LYS129, PRO130
WP_011669900.1	467.52	735.29	0.83	GLU85, LEU86, VAL87, LEU89, VAL90, VAL91, LEU93, THR97, GLU100, ILE101, LEU102, VAL104, CYS105, PHE108, ALA110, ILE112, LEU120, THR121, ILE122, TYR124, SER125, GLY126, ASN127, SER128, LYS130, VAL131, ALA133, ILE134, GLU136, ILE137, MET138, LYS140, TYR141, GLY142, VAL143, ILE146, ARG148

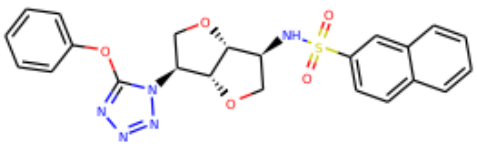
Supplementary table 5. Top 10 molecules from virtua screening for each identified target.

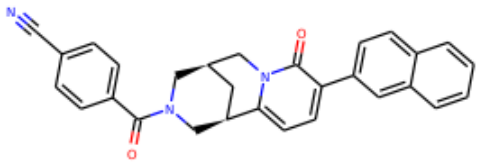
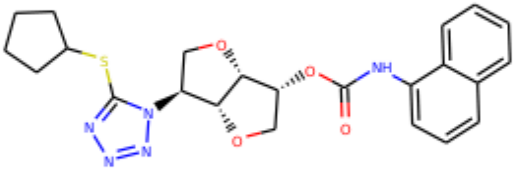
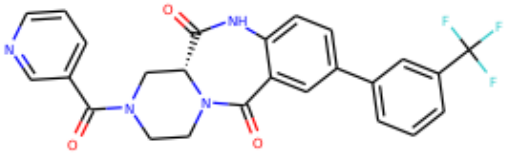
ZINC Compound ID	Score Binding Affinity	Number of H-bond	Active Residues
TIGR00730 family Rossman fold protein (WP_011671105)			
ZINC04235924	-6,5	1	ASP62
ZINC04237087	-7,4	0	-
ZINC04258800	-6,3	2	ARG88/ ARG101
ZINC04258868	-7,1	1	SER72
ZINC04259491	-6,6	2	LYS108/ LYS82
ZINC04259703	-6,9	3	LYS108/ SER109/ SER109
ZINC04270981	-7,1	1	SER72
ZINC05410189	-6,4	2	THR121/ ARG101
ZINC05415069	-6,7	1	ARG101
ZINC20503524	-7,1	3	GLY120/ THR 121/ ASP62
Cell division protein FtsZ (WP_011671028)			
ZINC04237101	-7,8	1	VAL229
ZINC04237102	-7,3	2	SER267/ ARG309
ZINC04237276	-7,5	2	SER10/ SER10
ZINC04259719	-9	2	PHE317/ ASP244
ZINC04270543	-8,6	2	SER267/SER267
ZINC05414969	-7,6	1	ASN193
ZINC05414981	-7,1	1	MET227
ZINC08297227	-8,1	1	SER267
ZINC20503175	-7,5	2	ARG196/ ARG309
ZINC67898728	-7	2	ASP189/ ASN248
Purine-binding chemotaxis protein CheW (WP_011670145)			
ZINC04235876	-7,4	1	GLU116
ZINC04235880	-8	1	THR120
ZINC04235884	-9,4	0	-
ZINC04237082	-7,8	1	GLU116
ZINC04237083	-7,7	1	GLU116
ZINC04237088	-8,2	1	GLU116
ZINC04237101	-9,4	0	-
ZINC08300249	-7,6	1	GLU116
ZINC08300250	-9,3	0	-

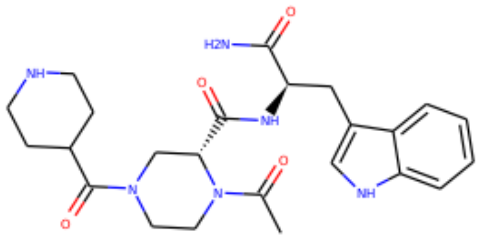
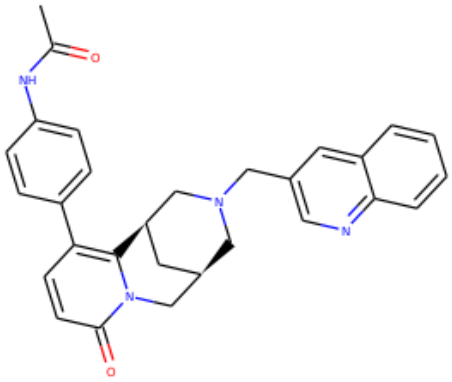
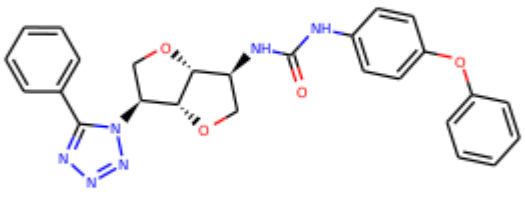
ZINC08300419	-8	1	PHE65
3-methyl-2-oxobutanoate hydroxymethyltransferase (WP_002754190)			
ZINC04260398	-8,4	1	ASN218
ZINC04235882	-7,6	1	ASN218
ZINC04235987	-7,8	2	LYS69/ ASN218
ZINC04258871	-8,2	1	LYS69
ZINC04237087	-7,2	0	-
ZINC04259385	-8	2	LYS69/ ASN218
ZINC04270981	-8,2	1	LYS69
ZINC04235924	-8	0	-
ZINC04236030	-7,5	2	LYS69/ ASN218
ZINC04235914	-8,2	1	LYS69
Uroporphyrinogen-III C-methyltransferase (WP_011669291)			
ZINC04235390	-7,6	1	-
ZINC04235906	-11	1	SER129
ZINC04235924	-10,8	0	-
ZINC04236030	-10,3	1	SER129
ZINC04259588	-9	4	GLY105/ ASP154
ZINC04260194	-7,6	1	-
ZINC05396462	-10,1	2	ASP100/ SER129
ZINC08295012	-8	2	GLY105/ ARG106
ZINC12605015*	-7,8	4	ASP154/ GLY105/ ARG106
ZINC20467419	-8,4	2	ASP49/ SER129
Imidazoleglycerol-phosphate dehydratase (WP_002750802)			
ZINC03842058	-6,9	0	-
ZINC04222703	-8,1	2	TYR127
ZINC04235908	-7,6	2	PHE125/ TYR127
ZINC04235924	-7,8	1	TYR127
ZINC04235940	-7,4	1	TYR127
ZINC04236020	-8,5	2	TYR127
ZINC04236030	-8,2	3	PHE125/ TYR127
ZINC15709489	-7,6	2	PHE125/ TYR127
ZINC31154666*	-7,4	4	GLU131/ TYR127/ TYR127/ GLN149
ZINC35415767	-6,9	2	PHE125/ GLN149
Acetolactate synthase small subunit (WP_011669900)			

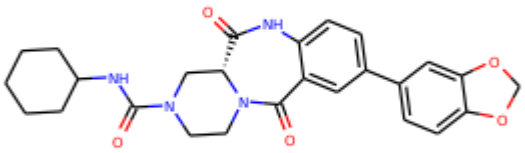
ZINC03841368	-5,5	1	MET138
ZINC03841468	-5,5	1	VAL143
ZINC04235924	-6,6	0	-
ZINC04235928	-5,8	1	LYS140
ZINC04235998	-6,2	0	-
ZINC04236001*	-6,8	1	SER125
ZINC04236003	-5,8	1	-
ZINC04236421	-6,5	0	-
ZINC05410504	-5,2	1	VAL143
ZINC06137732	-5,5	0	-
SsrA-binding protein (WP_011670499)			
ZINC04235846	-7,5	2	ASP51/ LYS17
ZINC04235852	-8,8	1	ASP151
ZINC04235880	-7,6	2	ASP51/ LYS17
ZINC04235908	-9,3	1	LYS55
ZINC04235913	-10	1	LYS55
ZINC04235914	-10,3	1	LYS55
ZINC04235916	-10,2	1	LYS55
ZINC04235924	-10,5	1	LYS55
ZINC04235937	-7,6	2	ASP51/ ASN15
ZINC15709489*	-8,1	2	ASP51/ LYS17

Supplementary Table 6. The chemical compounds with name, formula, and structure of each compound are shown according to their ZINC database identification number (ID).

ZINC ID	Compound name (IUPAC)	Molecular formula	Structure
ZINC4259719	N-[(3S,3aR,6S,6aR)-6-(5-phenoxytetrazol-1-yl)-2,3,3a,5,6,6a-hexahydrofuro[3,2-b]furan-3-yl]naphthalene-2-sulfonamide	C23H21N5O5S	

ZINC04237088	4-[(1R,9S)-5-naphthalen-2-yl-6-oxo-7,11-diazatricyclo[7.3.1.02,7]trideca-2,4-diene-11-carbonyl]benzonitrile	C29H23N3O2	
ZINC04259385	[(3S,3aR,6R,6aS)-3-(5-cyclopentylsulfanyltetrazol-1-yl)-2,3,3a,5,6,6a-hexahydrofuro[3,2-b]furan-6-yl] N-naphthalen-1-ylcarbamate	C23H25N5O4S	
ZINC15709489	(4aR)-3-(pyridine-3-carbonyl)-9-[3-(trifluoromethyl)phenyl]-2,4,4a,6-tetrahydro-1H-pyrazino[2,1-c][1,4]benzodiazepine-5,11-dione	C25H19F3N4O3	

ZINC12605015	(2R)-1-acetyl-N-[(2R)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl]-4-(piperidine-4-carbonyl)piperazine-2-carboxamide	C24H32N6O4	
ZINC20503524	N-[4-[(1R,9S)-6-oxo-11-(quinolin-3-ylmethyl)-7,11-diazatricyclo[7.3.1.02,7]trideca-2,4-dien-3-yl]phenyl]acetamide	C29H28N4O2	
ZINC31154666	1-[(3S,3aR,6S,6aR)-6-(5-phenyltetrazol-1-yl)-2,3,3a,5,6,6a-hexahydrofuro[3,2-b]furan-3-yl]-3-(4-phenoxyphenyl)urea	C26H24N6O4	

ZINC04236001	(4aR)-9-(1,3-benzodioxol-5-yl)-N-cyclohexyl-5,11-dioxo-2,4,4a,6-tetrahydro-1H-pyrazino[2,1-c][1,4]benzodiazepine-3-carboxamide	C26H28N4O5	

CONSIDERAÇÕES FINAIS

Neste estudo, foram criadas formulações de meio de cultura para isolar leptospiras, da urina de bovinos naturalmente infectados, pertencentes a dois genótipos do sorovar Hardjo (Hardjobovis e Hardjoprajitno). Vale ressaltar que os componentes adicionais foram úteis para o crescimento inicial (piruvato de sódio e soro fetal bovino) e subsequente manutenção de leptospiras (superóxido dismutase) mais fastidiosas. Com essa estratégia, utilizando três formulações, conseguimos isolar três linhagens puras do sorovar Hardjo. Após o isolamento, a técnica de MLVA associada ao sequenciamento parcial do gene *secY* foi eficiente para caracterização molecular de sorovares como Hardjo, que podem pertencer a diferentes espécies. Além disso, uma avaliação das células leptospiras nas três formulações por microscopia eletrônica mostrou diferenças na morfologia da espiroqueta com base no suplemento utilizado em cada meio.

Este estudo utilizou o sequenciamento de nova geração para sequenciar o genoma dos isolados dos dois genótipos do sorovar Hardjo, e assim realizar análises de genômica comparativa com intuito de buscar novos alvos vacinais e drogáveis através de ferramentas de bioinformática. Após a caracterização e localização subcelular das proteínas pelo software SurfG, esses genes foram direcionados para a Vacinologia reversa e para a busca por novos alvos de drogas através do docking molecular. De todas as proteínas predistas como potencialmente expostas à superfície (PSE) e de membrana, 17 foram classificadas como boas candidatas a alvos vacinais e classificadas no software Vaxign de acordo com as estruturas de transmembrana, capacidade de adesão ao MHC-I e MHC-II e densidade de epítopos. Entre os possíveis alvos da vacina, 10 foram proteínas não caracterizadas, duas lipoproteínas, duas proteínas do domínio Ig / proteína do domínio IPT / TIG, uma hidroxineurosporeno sintase CrtC, uma proteína motora flagelar MotB e uma proteína da família PF07603. Destas, as lipoproteínas são consideradas um importante fator de virulência e as proteínas não caracterizadas podem ter funções de adesão que também estão relacionadas à virulência das leptospiras, sendo assim, boas candidatas aos alvos de vacina. Com base na alta probabilidade de adesão, densidade de epítopos e características de virulência, sugere-se que cinco alvos podem ser bons candidatos imunogênicos e podem ser testados rapidamente em novas formulações de vacinas e posteriormente testados *in vivo*, representando uma

nova visão na prevenção e controle da leptospirose.

Para novos alvos de drogas foi utilizado o software Vina Autodocking e Chimera para encontrar os melhores alvos e ligantes de acordo com os *scores* de ligação (baixa energia) e número de ligação de hidrogênios em resíduos/ aminoácidos ativos. Com essas analises foi identificado oito possíveis alvos drogáveis, entretanto uma proteína teve maior destaque (proteína de divisão celular FtsZ), pois sua estrutura molecular 3D teve a melhor qualidade (>=50%), é considerada essencial para sobrevivência e está ligada a virulência da bactéria. O composto, produto natural ZINC04259719, foi o que teve melhor interação com a proteína FtsZ alvo, com menor energia e com mais ligações de hidrogênio ao se ligar aos sítios ativos da proteína e consequentemente, inibindo sua função. Esses alvos foram comparados com diferentes bancos de dados por meio do docking molecular e devem ser submetidos a análises futuras como possíveis recursos no combate ao estado de portador renal.

Todos os dados destacam que este estudo tem grande relevância para a saúde pública e animal, pois através da abordagem *in silica* foi possível encontrar novos alvos terapêuticos e vacinais para a leptospirose. Esses alvos podem ser testados rapidamente em novas formulações de vacinas e testes de drogas, representando um avanço na área da microbiologia médica. Além disso, outros estudos devem ser realizados com outras espécies de leptospiras, de acordo com a disponibilidade de genomas completos para obtenção de alvos vacinais universais.

PERSPECTIVAS:

Associado a este estudo gênomico, também foram realizadas analises de proteômica e metabolômica das leptospiras nos três diferentes meios de cultura (A, B e C) usado no isolamento de sorovares fastidiosos (dados em processamento). A partir dessas análises espera-se elucidar alguns pontos sobre a biologia e fisiologia das leptospiras em condições diversa de nutrientes e principalmente avaliar as diferentes proteínas e metabólitos produzidos pelas duas espécies (*L. interrogans e L. borgpetersenii*). A proteômica e metabolômica são recursos que combinados com as análises genômicas podem contribuir nas pesquisas sobre a patogenicidade e fatores de virulência das leptospiras.

ANEXO 1

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 026715 6

Dados do Depositante (71)

Depositante 1 de 1

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Tipo de Pessoa: Pessoa Jurídica

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