UNIVERSIDADE FEDERAL DO PARANÁ

DEBORAH CARBONERA GUEDES

DEVELOPMENT OF NEW BIOMOLECULES AS PROMISSING ANTIGEN CANDIDATES FOR LEISHMANIOSES DIAGNOSIS AND VACCINES

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Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Engenharia de Bioprocessos e Biotecnologia.

Orientadora: Profa. Dra. Vanete Thomaz Soccol

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RESUMO

As doenças tropicais negligenciadas (DTNs) são um grupo de doenças infecciosas que predominam em 149 países de regiões tropicais e subtropicais, afetando mais de um bilhão de pessoas. No Brasil, as DTNs como a leishmaniose, doença de Chagas, dengue e tuberculose estão presentes em quase todo o território, principalmente nas regiões com menor índice de desenvolvimento humano (IDH). As DTNs são responsáveis por consequências sociais e econômicas que as definem como doenças prioritárias na prevenção e no controle. Inserido neste cenário, o nosso grupo de pesquisa vem trabalhando no campo do desenvolvimento e aperfeiçoamento de técnicas de diagnóstico e profilaxia para as diferentes DTNs. Alguns destes trabalhos realizados demonstraram importantes e promissores resultados na avaliação de peptídeos miméticos como antígenos para o diagnóstico e vacina de leishmaniose. Algumas hipóteses foram levantadas e as quais serão respondidas neste trabalho. Em um primeiro momento, este trabalho convergiu para o diagnóstico de leishmaniose propondo o aperfeiçoamento do teste cutâneo. Estabeleceu-se um novo modelo animal para ser utilizado no controle de qualidade da produção dos antígenos do teste cutâneo, uma vez que nem sempre há coincidência entre o estágio das infecções em pacientes e a etapa de produção. O modelo animal proposto foi *Cavia porcellus* (porquinho da índia) devido à sua habilidade em reproduzir resposta imune similar a dos humanos. Para validar os resultados obtidos com os porquinhos da índia, o mesmo desenho experimental foi realizado em *hamsters*. Os resultados dos testes de imunização com as duas espécies animais validaram o modelo *C. porcellus* para o teste cutâneo, e demonstraram que eles possuem forte potencial como modelo *in vivo* para o controle de qualidade de produção dos antígenos do teste cutâneo. Em seguida, com base em bons resultados previamente obtidos com três peptídeos miméticos selecionados por *phage display,* e quimicamente sintetizados como moléculas solúveis, avaliou-se o potencial dos mesmos como antígenos no teste cutâneo. Os peptídeos, individualmente (PA1, PA2 e PA3) ou em conjunto (PAMix), foram testados em modelo animal (*C. porcellus*) imunizado com duas espécies de *Leishmania* (*Leishmania amazonensis* ou *Leishmania braziliensis*) e comparados com o antígeno referência do teste cutâneo. Os resultados mostraram que os peptídeos, individualmente ou em conjunto, foram capazes de promover reações de indução em 48 e 72 h após a realização do teste. Nos animais imunizados com *L. amazonensis* o melhor resultado foi alcançado pelo antígeno PA3, o qual foi superior ao do antígeno referência. Nos animais imunizados com *L. braziliensis* dois antígenos peptídicos apresentaram resultados satisfatórios, o antígeno PA2 e o PAMix, promoveram reações de indução com efeito mais prolongado do que o antígeno padrão. Estes resultados reforçam a hipótese de que os peptídeos podem ser utilizados como antígenos no teste cutâneo, e poderiam substituir o antígeno atualmente utilizado no diagnóstico de leishmaniose cutânea. Nova hipótese é levantada acerca da habilidade destes peptídeos em promoverem resposta imune celular e o seu potencial para serem aplicados como antígenos vacinais para a leishmaniose. Alinhado a isso, sugere-se que a capacidade imunogênica do antígeno poderia ser aperfeiçoada misturando os peptídeos já sintetizados e testados (PA1, PA2 e PA3), os quais são epítopos gerados a partir de proteínas da membrana do parasita, com peptídeos miméticos derivados de proteínas intracelulares do parasito. Com base nisso, a segunda etapa deste trabalho objetivou a pesquisa de epítopos derivados de histonas e sua avaliação *in silico* e *in vitro*. Pela análise *in silico* foi possível selecionar 11 peptídeos miméticos como moléculas imunogênicas. Destes, três (P_H31, P_H202 e P_H293) foram selecionados pelo ensaio de ELISA indireto e encapsulados em

lipossomos em conjunto com os peptídeos PA1, PA2 e PA3. O conjunto destes peptídeos foi analisado em testes biológicos como candidatos antigênicos. Primeiramente foram imunizados coelhos para avaliar a produção de anticorpos para confirmar a antigenicidade. Uma vez confirmada a imunogenicidade da quimera dos antígenos foi realizada infecção *in vitro* com macrófagos tratados com o conjunto de peptídeos, a qual demonstrou que os peptídeos foram capazes de induzir a síntese de IL-12, IFN-γ e IL-4. Os resultados demonstraram que para IL-12 os peptpideos aumentaram a taxa de produção em 5,64 (48 h) e 1,6 (72 h) em relação ao grupo controle negativo e quando infectados com *L. braziliensis*. Para *L. amazonensis* as taxas foram de 3,08 (48 h) e 4,34 (72 h). No caso IFN-γ as taxas foram de 0,27 (48 h) e 0,95 (72 h) nas infecções com *L. braziliensis*, sendo as taxas nas infecções com *L. amazonensis* de 1,81 (48 h) e 1,94 (72 h). Para IL-4 as taxas aumentaram em 1,46 (48 h) e 2,38 (72 h) para infecções com *L. braziliensis*, e 0,60 (48 h) e 2,38 (72 h) para *L. amazonenses*. Estes resultados demonstram que os peptídeos foram capazes de induzir a produção de um perfil de citocinas que conduz a uma resposta imune Th_1 e Th_2. Estes resultados também sugerem que os peptídeos são capazes de mimetizar proteínas do parasito que apresentam importante função na interação parasito-hospedeiro, e consequentemente poderiam ser usados para vacinas e para imunoterapia.

Palavras-chave: 1. Doenças tropicais negligenciadas 2. Diagnóstico 3. Peptídeos 4. Vacina 5. Leishmaniose

ABSTRACT

Negleted tropical diseases (NTDs) are a group of infectious illnesses that prevail in 149 tropical and subtropical countries affecting more than one billion of people. In Brazil, NTDs such as Leishmaniasis, Chagas diseases, dengue fever and turberculosis are present in over almost the entire territory, mainly in the regions with lowest human development indices. NTDs are responsible for social and economic consequences defining them as priorities for prevention and control. On this scenario, our research group has been working on the fields to improve and develop diagnosis and prophylaxis techniques for the different NTDs. Some of these previous works demonstrated great and promising results on the evaluation of mimetic peptides as antigens for leishmaniasis diagnosis and vaccines. Their results raised some hypotheses, which concern the focus of this work. At a first moment, this work focused on leishmaniasis diagnosis by proposing improvements for skin tests. The search looked to stablish a new animal model to be used on the quality control of skin test antigen production. The animal model proposed was *Cavia porcellus* (guinea pigs) due to its ability on reproducing a similar immune response to humans. To validate the results obtained with guinea pigs the same experimental design were tested in hamsters. The results from immunization tests with the two animal specie validated *C. porcellus* as a good model for skin test. The results also showed that they have strong potential as an *in vivo* model for quality control of skin test antigen production. Afterwards, based on good results previously obtained with three mimetic peptides selected by phage display and chemically synthesized as soluble molecules, it was evaluated their potential as antigens on skin test. The peptides, individually (PA1, PA2 and PA3) or in a mix (PAMix), were tested on animal model (*Cavia porcellus*) immunized with *Leishmania amazonensis* or *Leishmania braziliensis* and compared to the standard skin test antigen. The results demonstrated that the peptides, individually or in a mix, were able to promote induration reactions at 48 and 72 h after the test was performed. In animals immunized with *L. amazonensis* the best result was achieved by PA3 antigen, which were higher than the reference antigen. In animals immunized with *L. braziliensis* two peptides antigens, PA2 and PAMix, presented satisfactory results. These peptides promoted induration reactions for longer period of time than the standard antigen. These results reinforced the hypothesis that peptides can be used as antigens in skin test and could replace the current antigen used for cutaneous leishmaniasis diagnosis. The results from these works raised new hypothesis concerning about the ability of these peptides to promote cellular immune response and their potential to be applied as antigens on leishmaniasis vaccines. Align to this, another hypothesis suggests that the antigenicity capacity of the antigen could be improved by mixing the already synthesized and tested peptides (P1, P2 and P3), which are epitopes generated by parasites membrane proteins, with mimetic peptides from parasites intracellular proteins. Based on these, the second part of this work focused on search for epitopes from histone proteins and the in silico and *in vitro* evaluation of them. By the in silico analysis was possible to select 11 mimetic peptides as immunogenic molecules. From that, three (P_H31, P_H202 and P_H293) were selected by indirect ELISA and were entrapped in liposome structures in a mix with peptides P1, P2 and P3. This chimeric molecule was analyzed in biological assays as antigens candidates. The *in vitro* infection analysis with peptides mix-treated macrophages demonstrated that the peptides mix is able to induce the synthesis of IL-12, IFN-γ and IL-4. The results showed that for IL-12 the peptides stimulated an increase fold of 5.64 (48 h) and 1.6 (72 h) in relation to negative control group in infections with *L. braziliensis*. For *L. amazonensis* folds were 3.08 (48 h) and 4.34 (72 h). In the case of IFN-γ the folds were

0.27 (48 h) and 0.95 (72 h) in infections with *L. braziliensis*, and in infections with *L. amazonensis* were 1.81 (48 h) and 1.94 (72 h). For IL-4 the increase folds were 1.46 (48 h) and 2.38 (72 h) in infections with *L. braziliensis*, and 0.60 (48 h) and 2.38 (72 h) for *L. amazonensis* infections. The peptides mix was able to induce a cytokine profile production that lead to a Th_1 and Th_2 immune response. These results suggest that they are able to mimic parasites proteins that present important role in host-parasite interaction, and consequently could be used for vaccines and immunoprophilaxies.

Keywords: 1. Neglected tropical diseases 2. Diagnosis 3. Mimetic peptides 4. Vaccines 5. Leishmaniasis

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LISTA DE ABREVIATURAS OU SIGLAS

DNA - Deoxyribonucleic acid
RNA - Ribonucleic acid
v/v - Volume per volume
w/v - Weight per volume
NTD - Neglected Tropical Diseases
WHO - World Health Organization
HDI - Human Development Indice
VL - Visceral leishmaniasis
CL - Cutaneous leishmaniasis
MCL - Mucocutaneous leishmaniasis
MS - Minitério da Saúde
PCR - Polimerase chain reaction
nPCR - Nested polimerase chain reaction
qPCR - Quantitative polimerase chain reaction
QT-NASBA – Quantitative nucleic acid sequence based assay
ELISA - Enzyme link immunosorbent assay
MST - Montenegro skin test
IFN-γ - Interferon gamma
IL - Interleukine
NK - Natural killer cells
TGF-β – Transforming growth factor beta
OPD - o-phenylenediamine dihydrochloride
PBS - Phosphate buffered saline

LISTA DE SÍMBOLOS

µL - Microliters
µm - Micrometers
% - Porcentage
mg - Micrograms
-ΔΔCt - Relative gene expression
® - Registered mark
g - G-force
g - Grams

SUMÁRIO

1 GENERAL INTRODUCTION

Negleted tropical diseases (NTDs) are a group of infectious illnesses that prevail in 149 tropical and subtropical countries affecting more than one billion of people. The World Health Organization (WHO) identified seventeen diseases and pointed the commom factors that characterize them as NTDs. These factors englobe aspects as burden the poorest populations without access to basic health services; most of them are chronic and present slowly development conditions that progressively become worse if untreated and undetected; can cause severe symptoms with long-term consequences and life-long disabilities; and can result in social problems for NTDs patients once they are stigmatized and excluded from society (WHO, 2012; Molyneux, 2013).

The NTDs were classified into three categories based on the emergence, control and drug availability. The first category includes the major diseases such as dengue fever and leishmaniasis, which are emerging and are not under control. The second category englobes the malaria, the tuberculosis and the schistosomiasis for which parasite burden remains despite the availability of a control strategy. On the third category are the diseases (chagas and leprosy) which control strategy is effective, burden rates are decreasing and already exist a plan for their elimination (Lindoso; Lindoso, 2009).

Among the diseases established by WHO as neglected, ten of them are present in Brazil. Leishmaniasis, chagas disease, dengue fever, leprosy and tuberculosis are present in over almost the entire territory, mainly in the regions with lowest human development indices (HDI). About 10,000 deaths are annually recorded for NTDs in Brazil, which aligned to highest disease incidence, are responsible for severity socioeconomic consequences, corroborating to define them as priorities for prevention and control (Brandão et al., 2017; Martins-Melo et al., 2018).

Recently, the WHO proposed five strategies to prevent and control NTDs. These strategies comprise preventive chemotherapies, case management intensification, disease vector control, provision of clean water, improved of sanitation, and measurement of veterinary public health (WHO, 2010). Since then, many efforts have been performed in the field of preventive chemotherapies and disease management and reinforce the needs to search and develop new strategies for NTDs diagnosis and prophilaxies.

According to this scenario our research group has been invested on biotechnological tools to develop new opportunities of diagnosis and vaccines for neglected diseases.

As the aim of this work is about leishmaniasis, from now we will focus on this theme.

2 LITERATURE REVIEW

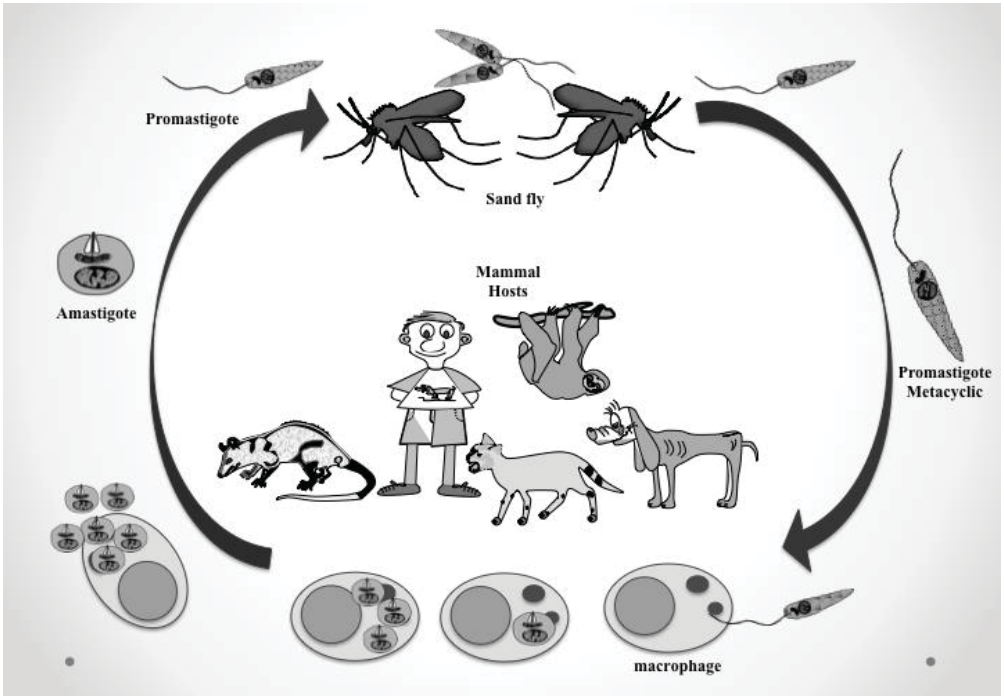
2.1 LEISHMANIASIS

Leishmanioses are a group of chronic diseases caused by the hemoflagellate protozoa parasite *Leishmania* spp. The protozoan *Leishmania* is characterize as an obligate intracellular parasite that multiples itself within the mononuclear phagocyte system of mammals. The disease is classified as one of the six major parasitic diseases groups, affecting 350 million people worldwide, and named as a neglected disease (Coura, 2005; Rey, 2008; WHO, 2015a).

Alexander Russel made the first clinical description of leishmaniasis, in 1756. In 1903, L.H. Donovan and W. B. Leishman independently analyzed autopsies of splenic tissue from patients infected in India and demonstrated the causative parasite. Ronald Ross, in the same year, proposed the name *Leishmania donovani* to the discovered parasite, christening the new genus *Leishmania* and the specie *donovani* (Bern; Chowdhury, 2006; Hide et al.; 2007). Actually, more than 30 specie were described and 10 infect human. Through the decades, the parasite evolved and becomes one of the major infectious diseases, affecting mainly the sub developing countries and poorest areas in the globe. The disease is reemerging and spreading due to some factors as malnutrition, depression of the human immune system environment changes and drug resistance (WHO, 2015b).

Leishmania parasites are transmitted to mammals through the bite of infected female phlebotomine sandflies during the blood meal. The sandflies inject the infective form of the protozoan (promastigotes), which are phagocytized by macrophages and dendritic cells. Inside these cells, the promastigotes transform themselves into amastigotes forms (Fig.1) that multiply by binary fission within the parasitic vacuoles on phagocytic cells' cytoplasm. The life cycle of *Leishmania* is completed when the amastigotes forms are ingested by the sandfly and are differentiated into promastigotes in the gut of the insect (Kima, 2007; Rey, 2008).

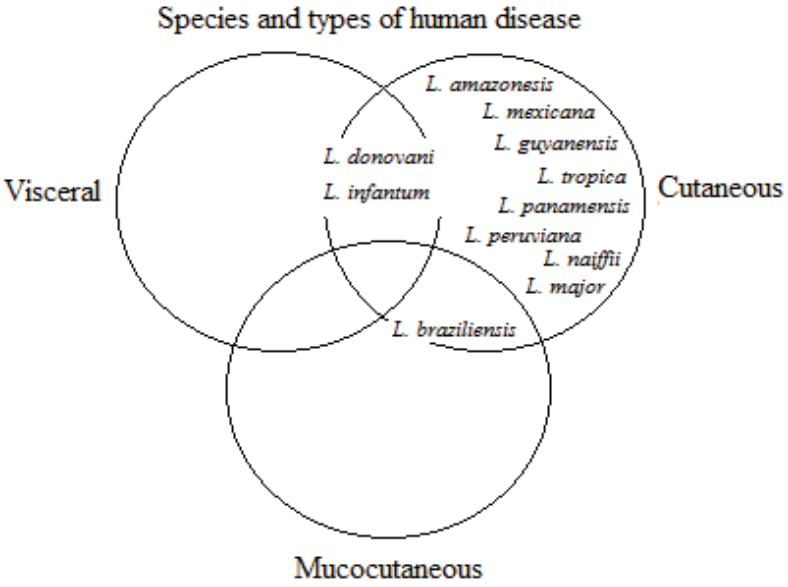
Figure 1: Biological life cicle of *Leishmania* spp. The transmission pathway, reservoirs and sandfly vectors of the parasite, showing the promastigotes and amastigotes forms.



Source: Adapted from Carreira et al., 2014.

The leishmanioses clinical manifestations develop after the mammal's host infection by the female sandfly bite. Several factors as the infective *Leishmania* specie, the inoculum quantities, the interaction between the parasite and the host, and the host immune response are necessary to develop the disease. Leishmanioses can manifest theirselves in three main forms: visceral leishmaniosis (VL); cutaneous leishmaniosis (CL or ATL in the Americas) and mucocutaneous leishmaniosis (MCL) (Brasil, 2013; WHO, 2015a). Fig. 2 gives the principal specie in the world and the disease form caused by them.

Figure 2: Principal *Leishmania* specie and the disease caused in mammals, especially in human.



The VL form (also known as Kala-Azar) is the most severe form of the disease and is caused by the complex *L. donovani* including two specie *L. (L.) donovani* and *L. (L.) infantum*. The parasites migrate to internal organs as spleen, liver, intestinal epithelial cells andbone marrow, resulting in symptoms as anaemia, fever, weight loss and spleen augmentation (Pinheiro et al., 2008). When not treated, the VL form can evolve to fatal endings in a rate of 100% within two years (WHO, 2015a).

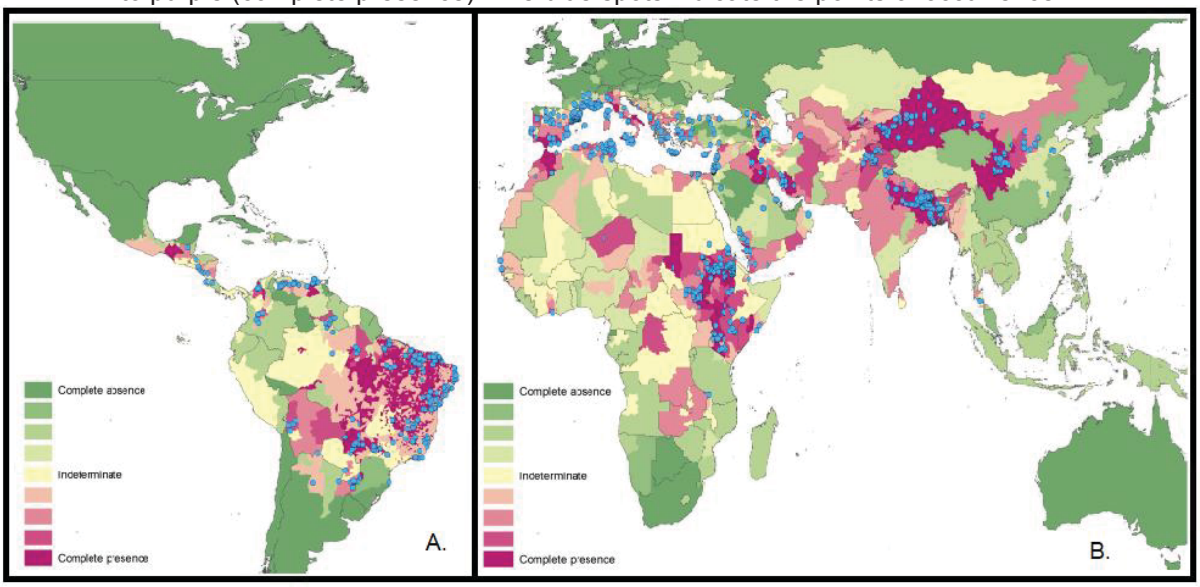
Cutaneous leishmaniosis (CL) is the most common and widespread form of the disease. This clinical manifestation causes epithelial lesions that develops itself within some weeks or months after the infection (in general three months). The lesions evolve initially from small papules or nodules to painless rounded open sores with a raised edge and central crater (ulcers). CL can manifest itself according to the skin lesions characteristics. It can be classified as diffuse, localized or disseminated cutaneous leishmaniosis. The disease is caused by several *Leishmania* specie and, the most important are *L. major* and *L. tropica*, in the Old World, and *L. braziliensis*, *L. amazonensis, L. guyanensis*, and *L. mexicana*, in the New World (Alves et al., 2013; Brasil, 2013).

Mucocutaneous leishmaniosis (MCL) is the severe form of CL, characterized by lesions in the mucous tissues of the throat, nose and mouth (Coura, 2005). In New

World, MCL is caused by *L. braziliensis*, but in some cases can be a sequela of an infection caused by parasites specie from cutaneous leishmaniosis (WHO, 2015a).

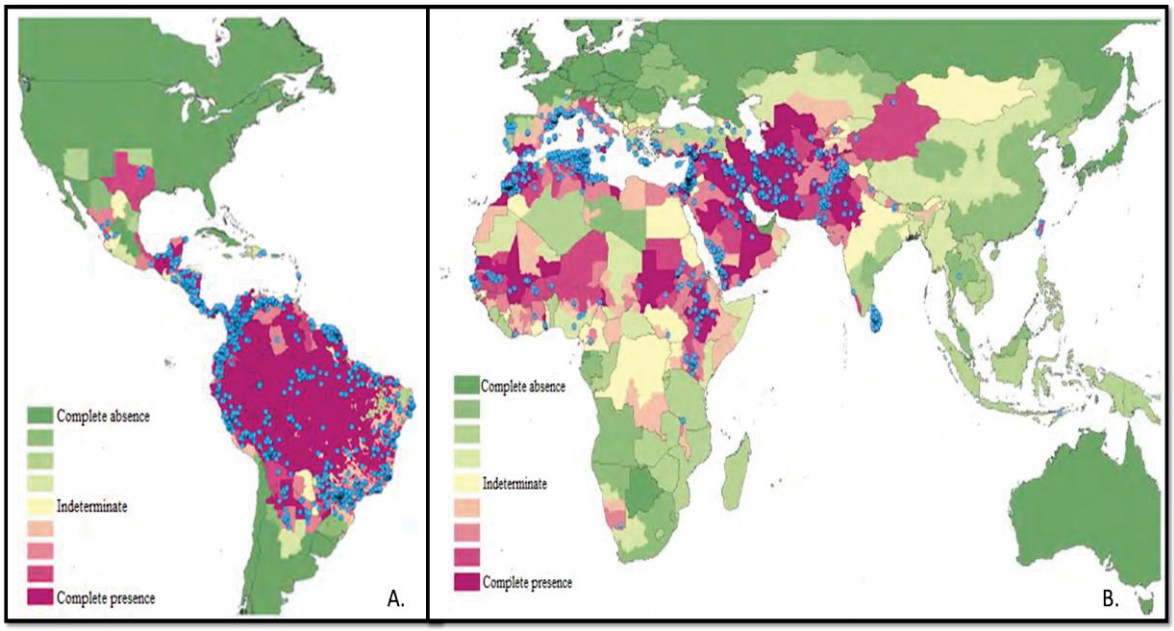
Leishmaniosis is endemic in 98 countries, being especially prevalent in Africa, Mediterranean basin, Western Asia and South America (Alvar et al., 2012). Approximately 1.3 million new cases are registered worldwide each year. Among these cases, 0.2 to 0.4 million are VL cases, and 0.7 to 1.2 million are CL. Almost 90% of global VL cases occur in six countries: Ethiopia, Bangladesh, Sudan, South Sudan, Brazil and India. Whereas cutaneous leishmaniosis is widely distributed with over than one-third of the cases registered in the four regions. The distribution of VL on the New and Old World is illustrated in Fig.3. The countries with highest occurrence of CL cases are Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru. The mucocutaneous form is mostly prevalent in South America and caused by *L. braziliensis* (Alvar et al., 2012; Pigott et al., 2014; WHO, 2015b). The main distribution of CL is illustrated in Fig.4 that presents the New World the Old World leishmaniosis distribution (Pigott et al., 2014).

Figure 3: A. Geografical distribution of visceral leishmaniosis in the New World. B. Geografical distribution of VL in the Old World. The presence of the disease ranges from green (complete absence) to purple (complete presence). The blue spots indicate the points of occurrence.



Source: Pigott et al., 2014.

Figure 4: A. Geografical distribution of cutaneous leishmaniosis in the New World. B. Geografical distribution of CL in Old World. The presence of the disease ranges from green (complete absence) to purple (complete presence). The blue spots indicate the points of occurrence.



Source: Pigott et al., 2014.

2.2 LEISHMANIASIS CONTROL

Leishmaniasis transmission occurs in a multiplicity of factors and complex biological systems. The diversity of vectors, reservoirs, parasites and epidemiological situations aligned to the lack of knowledge of these aspects stand out the complexity in disease control. Therefore, an efficient control and prevention of leishmaniasis are difficult and require a combination of mediation strategies. The current strategies, recommended by WHO, as control and prevention measures consist on early diagnosis and effective treatment, control of animal reservoir hosts and vectors, social mobilization and effective disease surveillance (WHO, 2018).

In Brazil, the Ministry of Health (Ministério da Saúde - MS in Portuguese) proposed that the prevention actions against CL might be flexible, specific and appropriate to each region or disease focus. On this case an epidemiological analysis regarding to entomological and parasitological studies to define the vactor and parasite specie, records of human cases and ecological studies to detect animal reservoirs, are required to determine the actions. The results of these analyses might focus in actions for early diagnosis and treatment, and to reduce the contact between

vector and humans by using insecticides, individual protection and reservoirs control (Brasil, 2013).

The individual protection measures recommended by MS include the use of thin-bed nets, screens on doors and windows, and chemical repellents. At risk areas, is suggests a security section of 200 to 300 meters between the urban area and the woods. For reservoirs control, when there are lesions identification, in case of domestic animals, exams are required to investigate the clinical aspects. In case of positive results for Leishmaniasis infection, the animal is maintained in clean areas away from human residences. A proper disposal of garbage is another important action for reservoir control, once organic matter accumulation and its improper packaging can attract reservoir animals with high levels of infection, which can contributes to disease incidence (Brasil, 2013).

Cutaneous leishmaniasis cases are defined as suspect, autochthonous and confirmed. Suspect cases are the ones when the patient presents cutaneous ulcers with granular bottom and an edge with a high frame. The conduct of a suspect case must be the clinical and epidemiological investigation, and if possible, the case must be submitted to diagnostic methods. If confirmed, the treatment is started according to technical standards by monthly monitoration up to three months after therapeutic coclusion (Brasil, 2013).

Autochthonous cases comprise confirmed cases which infection probably occurred in the patients' residence place. For a CL case to be considered as confirmed it is necessary to attempt for at least one of the following criteria:

- Residence, origin or displacement in areas with confirmed transmition; and parasite presence in direct or/and indirect parasitological examinations;
- Residence, origin or displacement in areas with confirmed transmition; and positive result in Montenegro skin test;
- Residence, origin or displacement in areas with confirmed transmition and without access to diagnosis methods.

In the case of VL, health authorities stablished preventive measures target to human population, vectors and canine population. In the case of humans, the measures to avoid transmition are related to individual protection such as thin-bed nets, screens on doors and windows, chemical repellents and access to areas of risk in crepuscular period. Concerning to the vector, the environmental sanitation is the

most important point for transmition control. On this case, permanent measures are required such as cleaning of yards, lands and public squares, disposal of organic waste and their proper destination and elimination of moisture source. These actions permit to alter the suitable conditions that allow the establishment of vectors' breeding sites. Regard to animals, the control is related to capture wandering dogs, use of screens in kennels to avoid sandfly entrance, and use dog-collar with Deltamethrin (4%) (Brasil, 2014).

The current tests available for leishmaniasis diagnosis are not accurate enough to be considered a gold standard. A good strategy is to combine different diagnosis techniques in order to obtain a more precise result. The first step is to make clinical and epidemiological diagnoses in cases where there is occurrence of typical leishmanioses symptoms (Brasil, 2013). In this case, to obtaining relevant information about the patient such as if s/he is coming from endemic areas or has been traveling to places where there are reported cases of leishmanioses. Then the patient is submitted to clinical diagnosis, to evaluate the symptomatology such as the examination of the lesions or check the presence of hepatosplenomegaly (Brasil, 2014; Brasil, 2013). However, it is necessary to confirm the diagnosis with laboratorial techniques due to the wide clinical spectrum of CL, which may mimic other skin conditions (fungal infection, sarcoidosis, mycobacterial ulcer, tropical ulcer). In the case of VL, this clinical manifestation can mimick autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, and autoimmune hepatitis) (Gomes et al., 2014; WHO, 2010; Tunccan et al., 2012). Due to this fact, the diagnosis results from a combination of epidemiological, clinical and laboratory data (Szargiki et al., 2009; Goto; Lindoso, 2010).

These main diagnosis techniques are based on the detection of humoral or cellular immune response to the infection and the presence of the parasite or its genetic material (Gomes et al., 2014; Skraba et al., 2015). The sensitivity of leishmaniosis diagnosis methods depends on factors such as the technical knowledge of the people responsible for perform the tests, the quality of the equipments, the evolution time of the disease, the clinical forms and the *Leishmania* specie involved in the disease (Brasil, 2013; Brasil, 2014).

Laboratorial techniques are useful tools to confirm the clinical diagnosis and can result in relevant epidemiological information through the identification of the parasite

specie, leading to actions to control the disease. This diagnosis comprises three main exams: parasitological, molecular and immunological approaches.

The parasitological diagnosis consists of exams that direct or indirect demonstrate the parasite (amastigotes on phagocytic cells' cytoplasm). Parasitological diagnosis is the reference test for CL detection due to its high specificity and with a variable sensitivity that depends on the stage of the lesion, the specie and the geographical location (Brasil, 2013; WHO, 2010). The material collected by these methods can be use for culture techniques, microscopic examination and molecular diagnosis (Daneshbod et al., 2011; Hosseinzadeh et al., 2012).

Molecular diagnosis is based on the detection of parasite nucleic acids (DNA) mainly by PCR-based methods. The molecular biology approaches present high sensitivity and specificity in comparison to other tests available being considered a promising technique (Silva et al., 2013; WHO, 2010). Different materials can be used as samples in molecular biology tests (biopsy, imprint of excised fragment, needle aspiration) to extract genetic material to be analyzed by this technique. The DNA amplification can be performing by different PCR-based techniques such as Nested-PCR (nPCR), Quantitative Real Time PCR (qPCR) and Quantitative Nucleic Acid Sequence- Based Assay (QT-NASBA) (de Paiva-Cavalcanti et al., 2015).

Immunological diagnosis is comprised by easy and accuracy methods which have been largely used as screening tools and as a definitive diagnosis methodology for leishmaniosis evaluation and other parasitological diseases. These methods are based on the detection of antibodies through different techniques such as immune fluorescence and Enzyme-Linked Immunosorbent Assay (ELISA). Despite of being a sensitive and specific method, immunological diagnosis of leishmaniasis can be cross-reactivity with other diseases, particularly to those caused by phylogenetically closely related specie, such as Chagas disease (Skraba et al., 2015; Souza et al., 2013). Another immunological diagnostic available for CL is the Montenegro skin test (MST), which is based in cellular response (Brasil, 2013; de Paiva-Cavalcanti et al., 2015).

2.3 LEISHMANIASIS VACCINES

The difficults regarding to leishmanioses raised the needs to develop prophilatic methods capable of induce protective immunity against the disease. According to

Gillespie and collaborates (2016) the development of an effective vaccine represents an assential measure to control and eliminate CL and VL. However, Thomaz-Soccol and collegues (2015) revising the patents and commercialized product showed that few vaccines are actually commercialized. But many patents are deposed specially in the United States (74 patents) that leads the ranking of patent applications for vaccines against *Leishmania*. On the sequence there is Brazil with 36 patents, which is an endemic region of leishmaniasis with 20,000 human cases of cutaneous leishmaniasis and over 3,000 cases of visceral form.

Nowadays, in Brazil, a law project approved in 2018 foresees the vaccination of animals against leishmaniasis on entire territory. This vaccination will be free and will be part of a national policy instituted to prevent and control the disease (Brasil, 2018). For human leishmaniasis, there is no currently licensed vaccine, although, several candidates have progressive to clinical trials (Gillespie et al., 2016). Since the WHO stablished the vaccine development as a goal in control and prevent leishmaniasis transmission, researches have been conducted on this field. These works aim to identify, isolate, characterize and purify important parasite molecules to be used as antigens in vaccination protocols against leishmaniasis (Nascimento et al., 2010; Chakravarty et al., 2011; Duthie et al., 2012; Coler et al., 2015).

The vaccines that are under development can be divided in three categories: live attenuated vaccines (incluiding genetically modified parasites); recombinant protein vaccines; and DNA vaccines (Coler; Reed, 2005; Jain; Jain, 2015; Mendonça, 2016). Leishmanization emerged as the most succesfull human immunization strategy permiting a long last protection against the disease induced by infection with live attenuated parasites (Costa et al., 2014a; Jain; Jain, 2015). Despite its use was interrupted in some regions due to the safety and drawbacks, this immunization is currently used in some countries against cutaneous Leishmaniasis (Saljoughian et al., 2014; De Brito et al., 2018). This scenario leads to the development of second generation vaccines, which apply synthetic or recombinant proteins derived from *Leishmania* antigens or parasites purified fractions (Costa et al., 2014a; Skwarczynski; Toth, 2016). Different studies regarding to the use of these antigens reported satisfactory results about the protection effectiveness and the possibility of their use as therapeutic or prophylactic vaccines (Coler; Reed, 2005; Baharia et al., 2015; Srivastava et al., 2016). The DNA/gene vaccines use parasites' genetic information,

and are produce by the insertion of genes, or genes fragments, that encode immunogenic antigens in plasmidial DNA or viral vectors (Costa et al., 2014a; Jain; Jain, 2015).

One of the reasons that an efficient vaccine has not yet been developed is because the disease is caused by different parasite specie that present particulary determinants of pathogenicity and virulence (Mendonça, 2016). On this scenario, the vaccine antigen must be recognized by different antibodies and be immunogenic for almost all of them (Martínez-Salazar et al., 2014). Current clinical trials of phase I and II have been performed for antigens derived from polyproteins expressed in bacteria registered as LEISH-F1 from The Infectious Disease Research Institute (IDRI) (ClinicalTrials.gov, 2013). The LEISH-F1 consists on three polypeptides that are present in *Leishmania donovani*, *L. chagasi* and *L. braziliensis*, which are infection agents of VL and CL, respectly. The vaccine phase I trials showed immunogenicity and safety in endemic and non-endemic populations. These results lead the IDRI to desing a new antigen, called LEISH-F2. The results from phase I confirmed the safety and immunogenicity of the therapeutic vaccine and progressed it to phase II trials to be test in a group of volunteers infected with CL. On the next step the safety, efficacy and immunogenicity will be test and compared to standard chemotherapy (ClinicalTrials.gov, 2015).

A new generation of vaccine has attracted the researcher's attention in the last years. That is the peptide vaccines, which permit to design more specific prophylatics with several benefits as good stability, absence of potentially damaging materials and antigen low complexity (de Brito et al., 2018). Several approaches have been performed to develop peptide-based vaccines such as peptide combination with polyepitopes, adenovirus and cellular vaccination. Recent advances point to vaccines design in chimeric, nano or polypeptide structures, which in a combination with antigenic peptides can be used to lead to a specific immune response (Kedzierska et al., 2012; Mahantesh et al., 2017).

Studies from Costa and collaborators (2014b) using phage display technique selected twenty phage clones, which were *in vitro* screened by immune stimulation of spleen cells from *L. infantum* BALB/c mice. The peptides able to induce specific Th1 immune response with high levels of IFN-γ and low levels of IL-4 were select to be evaluated as vaccines in BALB/c infected with *L. infantum*. The results from

vaccination showed that peptides were able to stimulate a high and specific production of IFN-γ and IL-12. They also show reduction in parasite burden in the liver, spleen and bone marrow. Toledo-Machado and collaborates (2015) selected an immunodominant peptide by Phage Display and demonstrated the ability of this peptide to promote a state of immunity against *L. infantum* infection in murine model. The results showed that this peptide is promising candidate for vaccine against VL in dogs.

A multi-epitope DNA vaccine proposed by Das and collaborators (2014) elicited strong immunogenicity in mice challenged with *L. donovani* promastigotes forms. The results also show that the vaccine promoted reduction in parasite load of spleen and liver. Another study used bioinformatics tolls to indentify among *Leishmania* proteins previously describe those able to generate immunogenic peptides. The results presented twenty-eight predicted peptides, which were agrouped in pools and were *in vitro* tested by stimulating peripheral blood mononuclear cells (PBMC) of patients from CL zoonotic region. It was identified that six peptides were able to stimulate the production of granzyme B, an important cytotoxic protein that mediates the apoptosis of target cells for cytotoxic $CD8^+$ lymphocytes and Natural Killing cells (NK), which constitute them as potential vaccine candidates (Naouar et al., 2016).

To support the development of vaccines against *Leishmania*sis, Freitas e Silva and collaborates (2016) applied an in silico analysis to identify potential epitopes belonging to *L. braziliensis* predicted proteome. With this test was selected peptide from proteins surface and conserved between *Leishmania* spp. The selected peptides were synthetized and *in vitro* tested to evaluate their ability to stimulate PBMC post treated CL patients. The results showed that the peptides were able to stimulate the proliferation of lymphocytes representing their immunogenic ability.

These studies show the promising applicability of peptides in CL and VL vaccines once they present attracting results regarding to the immune response, by trigging desired and important molecular markers for protection against *Leishmania* spp. Besides that, peptide vaccines showed the ability to reduce lesion size and parasite load in liver, bone marrow and spleen, which reinforce efficacy and potency use of this vaccine approach (Yang; Kim, 2015; Amit et al., 2017; de Brito et al., 2018).

2.4 RESEARCHES OF MIMETIC PETIDES AS ANTIGEN CANDIDATES FOR LEISHMANIASIS DIAGNOSIS AND VACCINES

Many efforts have been performed in the field of prophylactic and therapeutic alternatives for leishmaniasis. Insert on this context our research group have been invested on biotechnological tools to develop and search for new biomolecules able to be applied as antigens in diagnosis and vaccines for leishmaniasis.

In a recent work Link and collaborators (2017) selected three clones by Phage Display using immunoglobulins for *L. braziliensis*. The peptides were identified, synthesized and inoculated, individually and in combination, in hamsters. The immunized hamsters produced antibodies which recognized different proteins from *L. braziliensis* soluble antigens. The sequences of these peptides were analyzed in a data bank showing homology with *Leishmania* glycoprotein, lipophosphoglycan and other hypothetical *Leishmania* proteins. Furthermore, these peptides were analyzed as antigens in indirect enzyme-linked immunosorbent assay (ELISA) against serum samples of CL patients. The specificity of the peptides varies between 92 to 100% showing the ability of these peptides for diagnosis. These results raised some question such as if these peptides can be used for research cellular immunity and if they could be used for intradermo reaction tests.

The results from Link and collaborators (2017) raised the hyphothesis if these peptides can stimulate and generate cellular immune response and if these molecules were suitable to be apply as antigens in leishmaniasis diagnosis and vaccines. According this study it aimed to answer these questions by investigating and analyzing these peptides in diagnosis and prophylaxis approaches.

The mimetic peptides were evaluated as antigen candidates in skin test, one of the most used tests on complementary diagnosis and on epidemiological investigation of CL cases. This method, also known as Montenegro skin test (MST), consists in a cellular immunological diagnosis, which is sensitive, simple, and low cost test being positive in 87 to 100% cases (Antonio et al., 2014).

Since the first formulation until today, MST went through several standardizations. However, it is still constituted of *Leishmania* spp. promastigotes forms, where some of them are integrity forms and other are sonicated forms originated from a pool of *Leishmania* strains or single strains. Furthermore, leishmanin

suspension is composed by *Leishmania* spp. that varies according to etiological agent from each region (Skraba et al., 2015; Sadeghian et al., 2013). Added to this, the production process poses difficulties production scale-up, that are time consuming and the products quality control is realize in CL human patients (Skraba et al., 2015).

These factors raise the questions if it would be possible to replace the actual production quality control (test in humans) for animal model and if the current antigen could be replace by peptides formulation. According to this, the first part of this study aimed to answer these two issues:

1) Replacement of actual production quality control model;
2) Replacement of current skin test antigen.

These studies were already published (Guedes et al., 2017; 2019) and the results from the investigation with mimetic peptides as antigen candidates raised a hyphothesis if they could be used as molecules for leishmaniasis vaccines. Based on the hypothesis that the three peptides, with high homology with parasites' membrane protein (Link et al.; 2017) can generate and stimulate cellular immunity (Guedes et al., 2018), we investigate new biomolecules originated from parasites' intracellular immunogenic proteins. We also analysed them in a peptide combination, with the three previous peptides, as vaccine antigen candidates. Two articles were published (for detail see the attachment 1 and 2). In the first step (attachment 1) we proposed that certain animal models reproducing a similar immune response to humans may be used in the quality control of Montenegro antigen production. For this, fifteen *Cavia porcellus* (guinea pigs) were immunized with *Leishmania amazonensis* or *Leishmania braziliensis*, and, after 30 days, they were skin tested with standard Montenegro antigen. To validate *C. porcellus* as an animal model for skin tests, eighteen *Mesocricetus auratus* (hamsters) were infected with *L. amazonensis* or *L. braziliensis*. After 45 days, they were skin tested with standard Montenegro antigen. The results showed that *Cavia porcellus* immunized with *L. amazonensis* or *L. braziliensis*, and hamsters infected with the same specie presented induration reactions when skin tested with standard Montenegro antigen 48-72h after the test. Conclusions: The comparison between immunization methods and immune response from the two animal specie validated *C. porcellus* as a good model for Montenegro skin test. The model showed strong potential as an *in vivo* model in the quality control of the production of Montenegro antigen.

In the second step (attachment 2) we studied the application of the mimetic peptides as antigens in a skin test that is a widely used tool in diagnostic evaluations to investigate cutaneous leishmaniasis (CL). The actual antigen (Montenegro skin test [MST] antigen) presents some difficulties that pertain to its manufacturing and validation. To contribute to overcoming this problem, we propose the application of new-generation molecules that are based on skin antigen tests. These antigens were obtained through biotechnology pathways by manufacturing synthetic mimetic peptides. Three peptides, which were selected by phage display, were tested as skin test antigens in an animal model (*Cavia porcellus*) that was immunized with *Leishmania amazonensis* or *Leishmania braziliensis*. The peptide antigens, individually (PA1, PA2, PA3) or in a mix (PAMix), promoted induration reactions at 48 and 72 h after the test was performed. The indurations varied from 0.5 to 0.7 cm. In the animals immunized with *L. amazonensis*, the PA3 antigen showed better results than the standard MST antigen. In animals immunized with *L. braziliensis*, two peptide antigens (PA2 and PAMix) promoted induration reactions for a longer period of time than the standard MST antigen. These results validate our hypothesis that peptides could be used as antigens in skin tests and may replace the current antigen for CL diagnosis.

Now the investigation of this third part of the study has as aimed search and development of new biomolecules using biotechnological and bioinformatics tools to select and synthesized them. Evaluation of these biomolecules as candidates for leishmaniasis diagnosis and prophylaxis and is described on article 3 (see below).

3 ARTICLE 3

IN SILICO AND *IN VITRO* EVALUATION OF MIMETIC PEPTIDES AS POTENTIAL ANTIGEN CANDIDATES FOR PROPHILAXIS OF LEISHMANIASIS.

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3.1 ABSTRACT

An attractive alternative as leishmaniasis prophylaxis is the peptide-based vaccines, which uses an antigenic epitope able to stimulate long lasting immune response against the pathogen. This approach is a promising strategy once it can even promote protection against *Leishmania* and is a potent therapeutic tool to treat the disease. Furthermore, they are easier to produce and are more stable than the other vaccines. Based on that and on hypothesis raised by previous researches, this work target to search for epitopes from histone proteins and the *in silico* and *in vitro* evaluation of them. Seven intracellular immunogenic proteins from *Leishmania* spp. were selected for this study and were spot synthesized by F-moc strategy resulting in 302 pentadecapeptides epitopes. These epitopes were immunodetected using patients' serum with antibodies for *L. braziliensis*, *L. infantum*, and 22 spots were selected as reactive. The amino acid sequences of these peptides were chemicaly synthesized in soluble phase and tested in indirect ELISA assay using human patients' serum with anti- *L. braziliensis* or anti- *L. infantum* antibodies. Three of them (P_H31, P_H202 and P_H293) presented satisfactory results. From these results it was produced a chimeric molecule by entrapping them and three other peptides previous selected by our group (P1, P2 and P3) in the same liposome structure. This chimeric molecule was used to immunize a group of New Zealand White rabbits to study the immunogenic capacity of this quimeric antigen. The antibodies produced were analyzed by indirect ELISA using *L. braziliensis* or *L. infantum* total protein extracts. The results demonstrated that the peptides were able to induce humoral immune response against *L. braziliensis* or *L. infantum*. Then the peptides (P1, P2, P3, P_H31, P_H202 and P_H293) were ALA-Scan to evaluate the recognition of peptide epitope by rabbit anti-peptide polyclonal antibodies and identifying the essential amino acids for biding antibody-peptide. The results showed that for P1, P3 and P_H293 the change of all amino acids from their sequence into alanine decrease their capacity for antibody binding. In the case of P2 the amino acids Glu6, Pro7, Asn8, Pro9 and Leu10 were not fundamental for peptides recognition by anti-peptides polyclonal antibody. The same is observed for Glu1 and Ala2 from P_H31. For $P_H202$the aminoacids Ser1, Leu2, Ala5, Ile6 and Ser15 are crutial residues for the immunogenicity of the peptide. Then the peptides mix-treated rabbits' macrophages were *in vitro* infected with *L. braziliensis* or *L. infantum* and the cytokines

expression profile was analysed by RT-qPCR. The quantification of interleukins showed that the peptide mix was capable to induce a cytokine profile, which presented an increase fold in comparison to the control group for all tested cytokines. On *in vitro* infections with *L. braziliensis* the IL-12 presented an increase fold of 5.64 (48 h) and 1.6 (72 h); for IFN-γ were 0.27 (48 h) and 0.95 (72 h); and for IL-4 were 1.46 (48 h) and 2.38 (72 h). Infections with *L. amazonensis* the increase fold were 3.08 (48 h) and 4.34 (72 h) for IL-12; 1.81 (48 h) and 1.94 (72 h) for IFN-γ; and 0.60 (48 h) and 2.38 (72 h) for IL-4. It also demonstrated that peptides mix are able to induce the synthesis of IFN-γ, IL-12, and IL-4 inducing a cytokine profile production that lead to a Th_1 and Th_2 immune response. These results suggest that the mix of peptides derived from histone and parasites membrane molecules were able to mimic parasites proteins that present important role in host-parasite interaction.

Keywords: Vaccines. Synthetic peptides. Interleukins. *In vitro* infection. *Leishmania*

3.2 INTRODUCTION

Leishmanioses are one of the six major parasitic diseases and classified as neglected affecting mainly the poor population from developing countries (Rey, 2008; WHO, 2015). The high incidence of Leishmaniasis cases in these regions is largely a result of deforestation of rural areas, environmental changes and unplanned urbanization. These facts facilitate the contact between humans and vectors, and the interaction with animal reservoir which difficult the formulation of efficient strategies to control the disease. Besides that, the current disease treatment is based on pharmaceuticals that consist in drugs that are associated with severe side effects, toxicity, lack of efficacy and which contribute to increase the parasite resistance (Kedzierski, 2010). These factors raise the needs of developing and study new strategies to control the disease such as the implementation of prophylactic and therapeutic approaches.

Vaccination is among of the most successful prophylaxis treatment ever developed and appears as one of the interventions with the greatest impact on collective health. Since the studies of Edward Jenner, in 1976, which introduced this method for the first time by successful vaccination against variola virus, the

vaccination becomes an important prophylactic strategy. Its relevance, on immunization against infectious diseases was consolidated by worldwide vaccine programs that lead to complete, or almost complete, eradication of most of these diseases in developing countries (Skwarczynski; Toth, 2016, Abbas et al., 2015). The essential basis of vaccination is to provide an attenuate or killed form of an infectious agent, or components of these microorganisms, that do not generate the disease but induce immune response to provide protection against the infection of live pathogenic agent. The efficiency of a vaccine depends on the microorganisms' properties of not interfere with the host immune response, not stablishing a latency and not exhibiting variations on the antigenicity (Abbas et al., 2015).

On this way, leishmanization emerged as the first effort to immunize individuals living in leishmaniasis endemic areas. The vaccination of these individuals consisted on injecting live and virulent *Leishmania* spp. promastigotes, and despite the safety and drawbacks, this immunization is currently used in some countries against cutaneous leishmaniasis (Saljoughian et al., 2014; De Brito et al., 2018). In Brazil, the first clinical trials of human's immunization against leishmaniasis occurred in the 1940s by the studies of Salles-Gomes (1939) and Pessoa and Pestana (1940) (Correa, 1941). In the 1970s, Mayrink et al managed new tests with immunoprophylactic for leishmaniasis, which resulted in the development of the therapeutic vaccine Leishvacin® (Mayrink et al., 1979). Since that, several different approaches have been tested for anti-*Leishmania* vaccines. The clinical trials with *Leishmania*'s candidate's vaccine can be classified in approaches that apply whole killed parasites, live attenuated *Leishmania*, recombinant *Leishmania* antigen fractions; and DNA/gene vaccines (Noazin et al., 2008; Roatt et al., 2012; Fiuza et al., 2013; Chamakh-Ayari et al., 2014; Costa et al., 2014a; Jain; Jain, 2015).

The classical immunization with whole pathogen is still the most common despite all the advances on vaccines field. This approach generally generates long lasting immunity, but it presents some concerns about the safety of this form of vaccination once the inactivation of the antigen may not be ideal and the organism can recovery its pathogenic state. It also can cause strong allergic and autoimmune responses generated by the use of contaminated parasites' culture medium. More than that, the lack of a standardized parasite culture interfering on its registration, the

vaccines are poorly defined and present variability in their potency rendering inconclusive results (Noazin et al., 2008; Skwarczynski; Toth, 2016).

The recombinant and DNA/gene vaccines are alternatives to overcome the limitations presented by the classical one. They are molecularly well defined and with the advantage of being potentially safer than those produced from live attenuated parasites.

The recombinant vaccines are formulated with purified or recombinant proteins, or by protein subunits, such as immunogenic molecules present on the surface of *Leishmania* parasites and/or from molecules related to parasites' virulence (Kedzierski et al., 2006; Skwarczynski; Toth, 2016). However, these vaccines also present some drawbacks and are still not safety. The limitation of these vaccines regard to their production relative to protein's purity, stability issues, large-scale protein expression difficulties, glycosylation and undesired immune reactions.

The DNA/gene vaccines use parasites' genetic information, and are produced by the insertion of genes, or genes fragments, that encode immunogenic antigens in plasmidial DNA or viral vectors (Costa et al., 2014a; Jain; Jain, 2015). The main advantage of this technology is the induction of antibodies' production and the cellular immune response similar to observed response for attenuated vaccines (Nagata et al., 2004). However, there are some limitations on this approach such as the risk of plasmid integration on host genoma which can generate mutations (Henke, 2002).

Despite all the advances in immunization field and different strategies to identify new and antigenic molecules, there is no vaccine antigen capable of stimulating high levels of immune response to protect individuals against the disease. More than that, the large number of *Leishmania* specie responsible for cutaneous and visceral form difficult the formulation of a vaccine capable to englobe the immunization of individual affected by these two forms of the disease (Maroof et al., 2012). According to this, it is necessary to search for new technologies in the development of vaccine antigen candidates capable of trigger desire immune response in individuals at risk regions and which production is simple, easy to reproduce and minimize the problems associated with antigens' biological contamination (De Brito et al., 2018).

On this scenario, an attractive alternative is the peptide-based vaccines that uses an antigenic epitope, which is able to stimulate long lasting immune response against the pathogen. This approach is a promising strategy once it can even promote

protection against *Leishmania* and is a potent therapeutic tool to treat the disease (Skwarczynski; Toth, 2016; De Brito et al., 2018). Furthermore, they are easier to produce and are more stable than the other vaccines.

Peptide-based vaccines present several benefits regarding to their production. The antigens are produced by chemical syntheses approache and reduce the problem with biological contamination; characterize the antigens as chemical molecules, similar to classical drugs; their production is reproducible, simple, cost-effective and fast; and low cost to scale-up (Joshi et al., 2014). Concerning to the immune response, these vaccines can be customizing to generate specific responses and can be combined to design a multi-epitopes, or multi-specific, antigens to target different *Leishmania* specie or immunogenic parasites' molecules present in different stages of their life cycle.

Despite all advantages this vaccine approach has some challenges such as to enhance the immunogenicity of the peptides. One of the strategies to overcome this challenge is to design a multi-epitope based antigen, which consist on incorporating multiple epitopes into the vaccine and allow better coverage of natural pathogen antigen diversity (Moyle; Toth, 2013; De Brito et al., 2018)

A vaccine capacity to be effective on promoting long-lasting cell mediated immune response also depends on the molecules used as antigens on the different vaccines production approaches. A variety of different virulent *Leishmania* molecules have been tested as antigens including gp63 (glycoprotein leishmaniolysin), SLA (soluble *Leishmania* antigen), LPG (lipophosphoglycan), histones and several other purified antigens of *Leishmania* (Khamesipour et al., 2006; Olivier et al., 2012; Chamakh-Ayari et al., 2014; Martínez-Salazar et al., 2014). Among these molecules, histones constitute potential vaccine candidates against leishmaniasis, which constitute structural proteins with importance in the organization and regulation of genes. There are four principal *Leishmania* histone classes H2A, H2B, H3 and H4 that constitute the basic unit of chromatin, the nucleosome (Requena et al., 2000). These molecules are highly conserved antigens, produced by various *Leishmania* specie, which are non-secreted, but are able to induce an intense immune response (Santarem et al., 2007). These proteins are release during the infection process after intracellular amastigotes are eliminating by active macrophages. Moreover, they are capable of modulate the host immune response due to the fact that they do not suffer

selective pressure by the immune response, unlike surface and secreted proteins of *Leishmania* (Chang et al., 2003).

Some studies have been develop regarding to the use of histones as vaccine antigen candidates demonstrating their potential use on this field. De Carvalho and collaborates (2003) demonstrated that *Leishmania* proteins H2A and H2B could lead to the production of IFN-γ when used in the pre-stimulation of mononuclear cells from cutaneous leishmaniasis patients. Another study of Wang and collaborates (2011) used different concentrations of histones H2A and H2B *in vitro* treatment of mouse macrophages, which were infected with *Leishmania major, L. braziliensis* and *L. Mexicana* promastigostes, and observed a decreased on infectivity of promastigotes. According to this, histones are presented as promising molecules to be screened for selecting immunogenic epitopes that can be applied as peptide antigens on vaccines.

The identification and choice of an epitope are crucial stages to develop a peptide based vaccine. As first analysis, it is necessary to map the whole protein of interest to identify suitable sequences that are able to induce strong and permanent cellular immunity response against *Leishmania* parasites. This epitopes can be in silico identified and analyzed by bioinformatics tools which focused on the prediction of T and B cells, identification of conserved *Leishmania* specie sequences and if the epitopes are exposed sequences on protein quaternary conformation (Herrera-Najera et al., 2009; Freitas et al., 2016). The second approach is the *in vitro* analysis of the epitopes by biotechnological and biochemical tools such as phage display and spot synthesis techniques (Pini et al., 2005; Rhaiem; Houimel, 2016).

The Phage display method consists on DNA recombinant technology in which a nucleic coding sequence of an exogenous peptide is genetic fusion in frame to a gene that encode a bacteriophage coat protein. It results in a hybrid protein that is expressed on phage surface, and which contains the encoded peptide as a fusion product. The phage displayed molecules with high affinity to the target (i.e. antibody) are selected by an *in vitro* approach called biopanning. Then the identified peptide can be chemically synthesized and used as an antigen for different purposes (Costa et al., 2014b; Toledo-Machado et al., 2015; Link et al., 2017).

Spot synthesis is a biochemical technique to build epitopes in which a large number of peptides can be synthesized, and simultaneously tested, to identify and characterized active sites of interest. On this method, amino acids with

fluorenylmethyloxycarbonyl (Fmoc)-protection are chemically added to a cellulose membrane using an automated spot peptide synthesizer until the molecule is completed (Frank, 2002). After the chemical synthesis, the spot synthesized peptides are biologically test to identify the reactive epitopes, which can be by using enzymes, proteins, serum or other molecules depending the research approach. In the case of selecting peptides for vaccines it is performed an immunoassay to select the epitopes, in which human or animal patients' serum, with immunoglobulins for the disease of interest, is applied to select the reactive peptides. Then, the selected peptides are chemically synthesized in a soluble phase and used as antigen for different purposes (Reineke et al., 2001). The spot synthesis technique can be also use as a complementary method for phage display or to screening a protein to identify its epitope (Liu et al., 1999).

Our research group has been developing studies to obtain biotechnological products for immune protection purpose of individuals against leishaniasis. In recent studies, it was selected and synthesized three peptides that showed potential application as vaccine candidate (Seger, 2014; Link et al., 2017). On this study, it was verified that the peptides when used as prophylaxis antigens can confer protection to animals infected with *L. braziliensis*. In another research, our group tested the peptides as antigens for cutaneous leishmaniasis diagnosis (Guedes et al., 2019) and verified that these molecules can respond better that the current antigen applied on the test and maintained the immune response for longer. These results show that peptides can recruit and maintain a desired immune response and indicate that they can be applied as antigens for immune prophylaxis purpose.

On this scenario synthetic peptides highlights as molecules that represent defined epitopes with unique regions highly conserved and active sites that enhance ligation specificity with the antibodies. Besides that, this methodology can contribute to develop vaccine candidates with potential for immune protection of individuals affected by different forms of the disease, once its formulation involves regions that are highly conserved among *Leishmania* specie. We expected to contribute to the technological advances of potential biomolecules for immun protection against leishmaniasis prospecting a product highly efficient (specificity and sensibility) and low cost in a way to contribute and support the poor population. More than that, we hope with this new

biotechnology put Brazil at the forefront of research and production of leishmaniasis vaccines.

Based on that, this work proposes the investigation of new active peptides derived from histone proteins of *Leishmania* spp. though the production of a chimeric molecule of them with the three peptides previously selected by our group. The biological activity of this chimeric molecule was investigated to verify their potential to recruit immune response satisfactory for a vaccine candidate.

3.3 OBJECTIVE

3.3.1 General objectives

Search and development of new biomolecules using biotechnological and bioinformatics tools to select and synthesized them and use these new biomolecules to produce a chimeric molecule, together with three other peptides previously selected, and evaluate it as a candidate for leishmaniasis prophylaxis.

3.3.2 Specific objectives

- Select intracellular immunogenic proteins from *Leishmania* spp;
- Spot synthesize the protein selected and immunodetect the reactive spots (mimetic peptides);
- *In silico* evaluate the immunogenicity properties of mimetic peptides;
- Evaluate peptides' reactivity against human patients anti *L. braziliensis* and anti *L. infantum* antibodies;
- Encapsulate the peptides in liposome structures (chimeric molecule);
- Production of rabbits' anti-peptides polyclonal antibodies;
- Evaluate IgG antibody production profile by indirect ELISA assay;
- ALA-Scan peptides evaluation with rabbits' anti-peptides polyclonal antibodies;
- *In vitro* experimental infection of macrophages from rabbits immunized with peptides quimera;

- Evaluation of interleukins' mRNA expression by real time PCR (RT-qPCR).

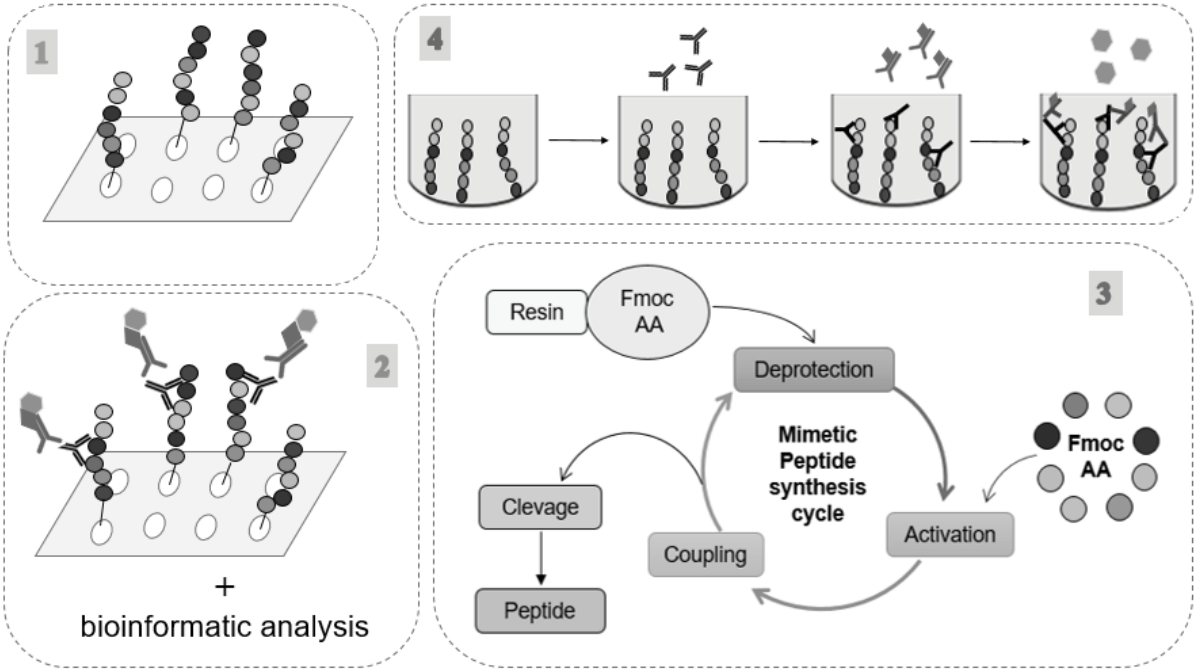
3.4 MATERIAL AND METHODS

The methodology of this work comprehends two major steps. The first one was divided in process of peptides' selection, synthesis and evaluation. The second one encloses experimental procedures to evaluate peptides' immunogenicity analyzing humoral and cellular immune responses.

3.4.1 Step 1 - Peptides selection: *in silico* and *in vitro* procedures

The figure 1 show all step realized to screening and select the antigenics peptides. Previously, seven intracellular immunogenic proteins from *Leishmania* spp were selected for this study, which were spot membrane synthesized (step 1) and the reactive peptides were select by immunodetection assay (step 2). The reactive spots were then analysed by bioinformatic tools to evaluate their chemical and structure charactheristics in order to verify protein regions that manifest epitope-like aspects (step 2). The peptides selected were chemically synthesized (step 3) and *in vitro* analyzed to estimate their reactivity against human patients' serum with antibodies for *L. braziliensis* or for *L. infantum* (step 4). The results from this test permited to assess and select the immunogenic peptides.

Figure 1: Experimental process for selection, synthesis and evaluation of the peptides. (1) Spot synthesis of the intracellular immunogenic proteins from Leishmania spp. (2) Immunodectection assay of reactive spots and their analysis by bioinformatic tools. (3).



3.4.1.1 Selection of intracellular immunogenic proteins from *Leishmania* spp.

Seven intracellular immunogenic proteins from *Leishmania* spp. were selected for this study. The proteins choices were Histones identified as HP1 (UniProt accession number: Q9NL78), HP2 (Q9NL77), HP3 (Q9BMY8), HP4 (A4H9W0), HP5 (A4HBV1), HP6 (O44009), and HP7 (A4HNK4). The proteins derived from *L. amazonensis* and *L. braziliensis*. The homology of the protein sequences among *Leishmania* spp. (*L. amazonensis*, *L braziliensis* and *L. infantum*) were verify by EMBOSS Needle Pairwise Sequence Alignment (Rice, 2000).

3.4.1.2 Proteins spot-synthetized peptides and immunodetection assay

To select and evaluate reactive epitopes from the proteins previously mentioned, their sequences were spot-synthesized by overlapping pentadecapeptides offset of three amino acids scanning. The Spot synthesis was performed on a cellulose membrane with fluorenylmethyloxycarbonyl (Fmoc)-protection using an automated spot peptide synthetizer (Intavis Bioanalytical Instruments, Nattermannallee, Germany) (Frank, 2002).

For immunodetection assay, the cellulose membrane was firstly blocked, for nonspecific binding, by overnight agitation with 3% (w/v) casein and 0.5% (w/v) sucrose dissolved in TBS-T (0.1% Tween 20 (v/v) in TBS) at 4°C. Afterwards, the membrane was washed with TBS-T 0.1% for 10 min under agitation, and probed, for 90 min at 37°C, with patients' serum with antibodies for *L. braziliensis*, *L. infantum*, or negative control diluted 1:100 in blocking buffer (3% (w/v) casein, 0.5% (w/v) sucrose and TBS-T 0.1%). The membrane was washed again and incubated with biotin-labeled secondary antibody (1:30,000) diluted in blocking buffer for 60 min at 37°C, followed by an incubation step with Streptavidin (1:10,000) diluted in blocking buffer for 60 min at 37°C. After two washes, positive spots were visualizing by ECL™ system.

3.4.1.3 In silico analysis of peptide sequences.

Protein sequences were also analyzed using bioinformatic tools such as Peptide 2.0 (https://www.peptide2.com/main_about.php), IPC (Kozlowski, 2016) and PepCalc (https://pepcalc.com/) to evaluate characteristics as molecular weight, isoeletric point, net charge and hydrophobicity. Other tools such as Epitopia server (Rubinstein, 2009), ABCpred (Saha; Raghava, 2006) and IEDB Analysis Resource (Vita, 2014) were used in order to verify protein regions that manifest epitope-like characteristics. Addionally, the 3D structures of proteins were obtained by a homology-modelling Server, SwissModel (Guex, 1997), using histones X-ray diffraction structure as templates (HP1 was 3le1.1; HP2 was 4zux.1; HP4 was 2rvq.1; HP5 was 1id3.1; HP6 was 5o9g.1; HP7 was 4jjn.1). The predicted epitopes were visualized and analyzed by Swiss-Pdb Viewer (Guex, 1997). Finally, the peptides sequence were BLASTed against *Leishmania* spp. protein sequences, using TriTrypDB to analyse the similarity between them (Aslett et al, 2010).

3.4.1.4 Chemical synthesis of selected peptides

The peptides selected by immunodetection assay and analyzed by bioinformatics tools were chemically synthesized according standard protocol by Fmoc strategy (9-fluorenylmethyloxycarbonyl) using a resin as insoluble solid support

(Merrifield, 1969) with MultiPep RS automated peptide synthesizer (Intavis Bioanalytical Instruments, Nattermannallee, Germany).

After the last synthesis cycle, the peptides were released from the resin by trifluoracetic acid treatment, filter and precipitate with cold ethyl ether, yielding the peptides. After centrifugation, the ether was discarded and peptides were lyophilized, weighed, dissolved in ultrapure water and stored at -20°C until the next step.

3.4.1.5 Peptides' reactivity against human patients anti *L. braziliensis* and anti *L. infantum* antibodies.

An indirect ELISA procedure was performed to evaluate the reactivity of peptides against human patients' serum with antibodies for *L. braziliensis* or for *L. infantum*, and to select the immunogenic ones. Different concentrations of antigen (peptides) and second antibody, and different dilutions of patients' sera were tested by combining them in distinct ways (Fig.2).

Figure 2: Indirect Enzyme link immunosorbent assay (ELISA) experimental design to evaluate the reactivity of peptides against patients' serum with antibodies for *L. braziliensis* and *L. infantum.* The columns 1 to 3 correspond to different concentrations.

	Serum dilution	1	2	3	Second antibody dilutions
A		+	+	+	
B	1:100	-	-	-	
C		+	+	+	1:7,500
D	1:200	-	-	-	
E		+	+	+	
F	1:100	-	-	-	
G		+	+	+	1:10,000
H	1:200	-	-	-	
		0.25 µg/ml	0.5 µg/ml	1 µg/ml	

Antigens concentrations

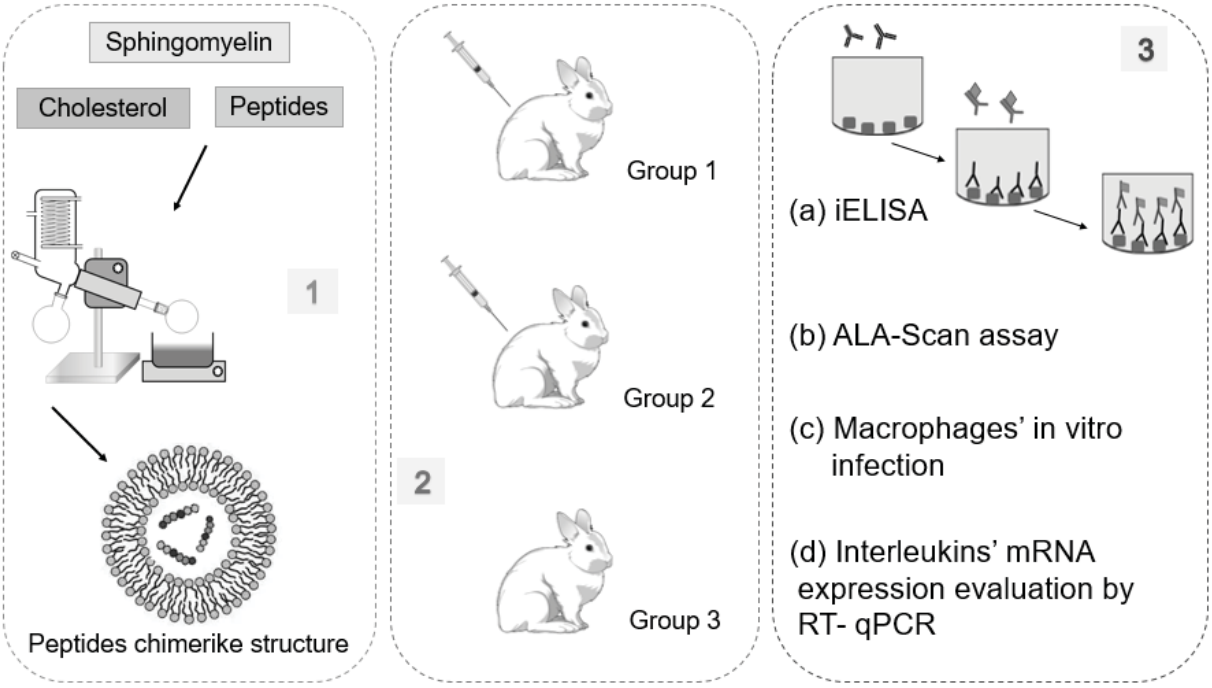
For iELISA assay microtiter plates were coated with 100 µl of each peptide diluted in carbonate buffer 0.05 M (pH 9.6) and incubated overnight at 4 °C.

Subsequently, the plates were wash with a solution containing 0.9% (m/v) of NaCl and 0.05% (v/v) of Tween 20, and blocked with a blocking buffer (casein 2% (m/v) dissolved in phosphate-buffered saline - PBS) for 1 h at 37°C. Then, the plates were washed, and incubated with human sera diluted in incubation buffer (0.25% (m/v) of casein, 0.05% (v/v) of Tween 20 in PBS pH 7.4) for 1 h at 37°C. After washed the plates, the antibody binding was determined by antihuman IgG (Fc specific) peroxidase antibody diluted in incubation buffer for 1 h at 37°C. The reaction was detecting by adding 100 µL of substrate buffer (2 mg of o-phenylenediamine dihydrochloride (OPD® - Sigma Aldrich) + citrate buffer (Na_2HPO_4 0.7% (m/v); $C_6H_8O_7$ 0.5% (m/v) in distilled water + 2 µL of H_2O_2). After 15 min, the reaction was stoped with 20 µL H_2SO_4 (5 % (v/v)) and the absorbance was measured at 492 nm.

3.4.2 Step 2 - Production and evaluation of peptides chimeric molecule.

The figure 3 summurizes the steps involved on the second part of this study comprising the production and evaluation of peptides chimeric molecule. First, the peptides selected on step 1 of this work and the peptides selected by previous work of our group, were encapsulated in liposomes to produce the chimeric molecule (Fig. 2 step 1). Then, these molecules were used to formulate the antigen for animal immunization to produce specific polyclonal antibodies (step 2), which activities were evaluated against *L. braziliensis* and *L. infantum* protein extract antigen (step 3). The anti-peptide polyclonal antibodies were also used in ALA-scan assay to identifying the essential amino acids for biding antibody-peptide (step 3). To verify if the chimeric molecule was able to induce humoral and cellular immune response, the blood and the macrophages of immunized animals were collected to evaluate the pattern of IgG and cytokines production during the immunization period and in a condition of experimental infection, by macrophages *in vitro* infection with promastigotes forms of *L. braziliensis* and/or *L. infantum*. The expression of mRNAs of interleukins and inducible nitric oxide synthase (iNOS) was evaluated by qPCR method (step 3).

Figure 3: Experimental process for production and evaluation of the peptides chimeric molecule. (1) Peptides' encapsulation in liposomes. (2) Rabbits' immunization with mix of six peptides (chimeric molecule) to produce anti-peptides polyclonal antibodies. (3) Evaluation of peptides' immunogenic capacity to induce immune response: (a) ELISA assay to evaluate IgG antibody production profile; (b) Evaluation of ALA scanning peptides with rabbits' anti-peptides polyclonal antibodies; (c) *In vitro* experimental infection of macrophages from rabbits immunized with peptides; (d) Evaluation of interleukins' mRNA expression by RT-qPCR.



3.4.2.1 Reactive peptides' encapsulation in liposome

The immunogenic peptides (selected by ELISA in this work – P_H31, P_H202 and P_H293) plus three peptides, selected by a previous work of our research group (P1, P2 and P3) (Link et al., 2017), were encapsulated in liposome according to Toledo-Machado and collaborates (2015), and so producing a six-peptide chimeric molecule. Briefly, the entrapped peptides were prepared by dissolving sphingomyelin (25 mg) and cholesterol (6.5 mg) in 5 mL of a solution containing methanol and chloroform (1:2). The solvent was removed by flash evaporation on a rotatory evaporator at 37 °C and dryed for 80 min under reduced pressure. After that, an aqueous phase containing the six peptides (500 µg of each peptide) diluted in 3 mL of PBS pH 7.4 was added to lipid film composing the liposomes structures with the mix of peptides encapsulated. To dislodge and retrieve the liposomes they were treated with three time's ultrasonic vibration of 20 s. To remove non-encapsulated peptides, the liposome suspension was

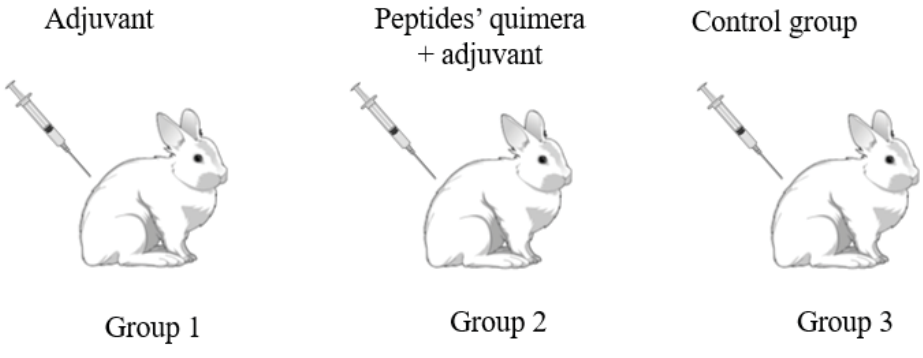
washed two times by centrifugation for 10 min, 8,000 *g* at 4 °C and resuspended in PBS pH 7.4. After washing, the liposomes were lyophilized and stored at 4°C.

3.4.3 Production of rabbits' anti-peptides polyclonal antibodies

For immunization procedure to produce polyclonal antibodies, it was used five adult female New Zealand White rabbits (NZW), weighing 2.1 - 2.6 kg. The animals were housed in single cages in a standard animal room (20°C and 55% humidity) and fed a balanced diet and water ad libitum. The animals were divided in groups: Group 1 – immunized with with the adjuvant (Aluminun hydroxide); Group 2 - immunized with peptides' mix entrapped in liposome; and Group 3 - without immunization (control group).

On the first immunization (Day 0), the Group 1 was intramuscular injected with 1 mL of aluminum hydroxide. Group 2 was intramuscular injected with 500 µg/ animal of entrapped peptides' mix dissolved in PBS buffer (pH 7.4) and 1 mL of aluminum hydroxide (adjuvant). The Group 3 was not immunized. The other immunizations were performed in same conditions on days 15, 30, 45, 60 and 90. On day 90 the animals were bleed, euthanized and macrophages were collected for *in vitro* assay (Fig.4).

Figure 4: Animals' immunizations were performed every each 15 days from day zero to 90.



Immunizations period = Days 0, 15, 30, 45, 60 and 90.

Blood samples for polyclonal antibody reactivity evaluation were obtained before every immunization (Days 0, 15, 30, 45 and 60), by vein puncture from the marginal ear vein of the rabbits and transferred to vacutainer blood collection tubes.

For interleukin evaluation, blood samples were collected at 24, 48 and 72 h after the first immunization (Day 0), transferred to vacutainer blood collection tubes containing 200 µL of Trizol. After each sample collection, they were centrifuged at 448 *g*, for 10 min, at room temperature, resuspended in Red Blood Cell (RBC) lysis buffer pH 7.3 (89.9 g of NH_4Cl; 10 g of $KHCO_3$; 2 mL of EDTA 0.5M), incubated for 5 min and centrifuged for 2 min, 1,008 *g* at room temperature. The supernatant was discarded, and the pellet was resuspended in PBS, centrifuged for 2 min, 1,008 *g* at room temperature and resuspended in 400 µL of trizol. The samples were stored at -80°C.

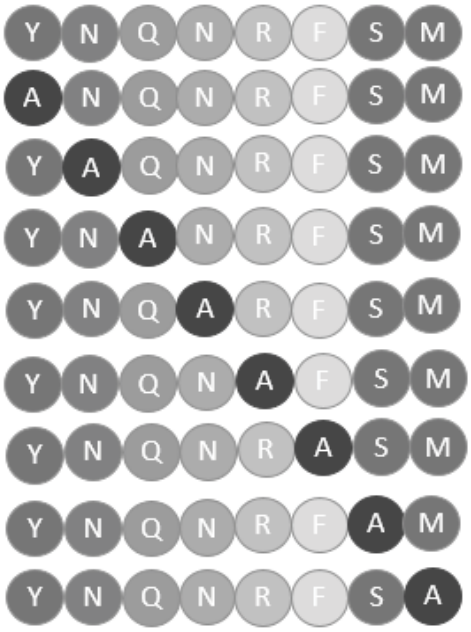
3.4.4 Evaluation of IgG antibody production profile.

An indirect ELISA assay was performed to evaluate the profile of the polyclonal antibodies reactivity obtained during rabbits' immunization period. The ELISA procedure was performed as described in item 2.5. The microtiter plates were coat with *L. braziliensis* or *L. infantum* protein extracts in a concentration of 1 µg/well. The rabbits' sera, with anti-peptide polyclonal antibodies, and the second antibody (anti-rabbit IgG (whole molecule) peroxidase – Sigma Aldrich) were respectively diluted 1:200 and 1:7,500 in plates coated with *L. braziliensis* protein extract, and 1:100 and 1:10,000 in plates coated with *L. infantum* protein extract.

3.4.5 Epitopes characterization by ALA scanning method.

Alanine analogues of the peptides were synthesized by Spot synthesis method to evaluate which amino acid, of each peptide sequence, was essential for the interaction with rabbit anti-peptides polyclonal antibodies. For ALA-Scan assay, the peptides were synthesized, in a cellulose membrane, by replacing each amino acid in the original sequence for an alanine at a time (Fig. 5). The immunoassay to assess the essential amino acids was performed as described in item 2.2. The rabbit anti-peptide sera were diluted 1:100 and anti-rabbit IgG (whole molecule) peroxidase antibody 1:10,000. The ALA scanning peptides were also immune tested with anti-*L. braziliensis*, anti-*L. infantum*, anti-*Trypanosoma cruzi* and anti-*Mycobacterium tuberculosis* antibodies.

Figure 5: Example of ALA scan synthesis for the six peptides. Each amino acid in the original sequence is replace for an alanine at a time.



3.4.6 *In vitro* experimental infection of macrophages from rabbits immunized with peptides quimera

3.4.6.1 Macrophages *in vitro* infection with L. braziliensis and L. infantum promastigotes forms

The parasites were obtained from cultures of *L. braziliensis* and *L. infantum* separately cultivated in biphasic brain heart infusion medium until stationary phage (5 days). The parasites were harvested from culture media, two times centrifuged with PBS for 15 min, 1000 *g* at 10°C, resuspended in RPMI + 20% FBS + 10% penicillin-streptomycin, and counted in Neubauer chamber. For the infection, 500 µL of parasites suspension were added to each well containing the adhered macrophage cells. The parasites were added in a proportion of 15:1 (parasites: cells) and were incubated at 37°C and 5% CO_2, for variable times (24, 48 and 72 h). Supernatant samples were collected at each assay time point (24, 48 and 72 h), and stored at -80°C until used for interleukin evaluation.

3.4.7 Evaluation of interleukins' mRNA expression by real time PCR (RT-qPCR)

A reverse transcriptase and quantitative real time PCR (RT-qPCR) was performed to assess the expression of mRNAs of interleukins and inducible nitric oxide synthase (iNOS) in samples from blood, collected during rabbits' immunization, and from supernatant of the *in vitro* infection cultures.

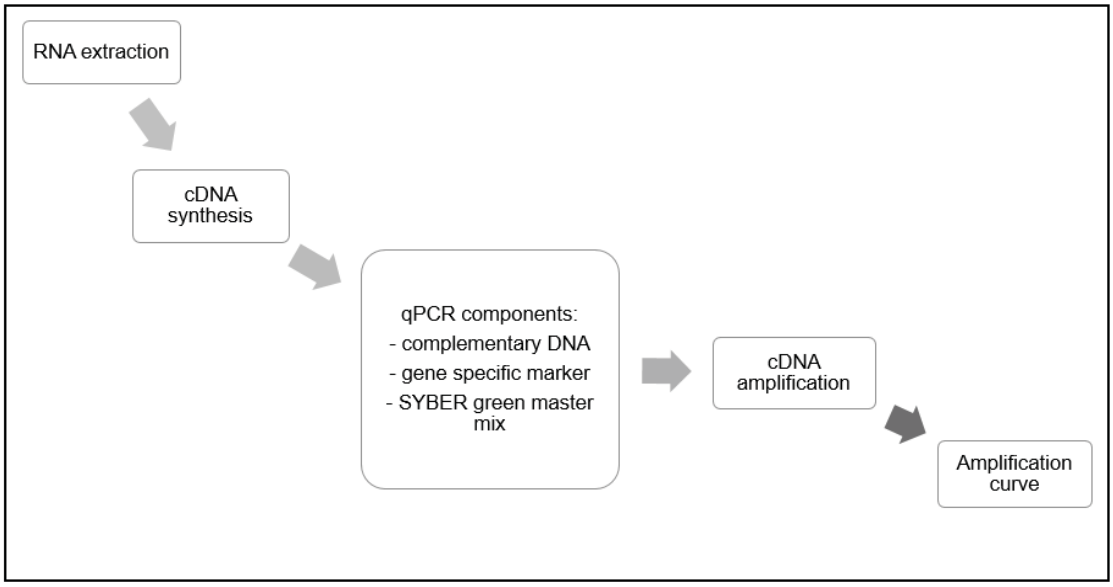
Total RNA of samples was extracted using RNeasy Mini Kit (QIAGEN®) based on manufacturer's instructions. To remove contaminating genomic DNA from RNA samples, it was used a TURBO DNAse free kit (Invitrogen®). The samples were quantified by spectophometers, and a total of 150 ng of isolated RNA was reverse transcribed to cDNA using a First Strand cDNA Synthesis kit (Thermo Scientific®) with oligos dT_{15}. Complementary DNA was mixed with 10 pmol of each gene-specific marker (Tab. 1 and Fig. 6) and 2.5 µL of SYBER green PCR master mix (Applied Biosystem). The mRNA expression of cytokines and inducible nitric oxide synthase (iNOS) was assessed using StepOnePlus Real Time PCR System (Thermo Scientific). Data analysis was performed by Livak method ($2^{-\Delta\Delta Ct}$) (Livak; Schmittgen, 2001) using the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) to normalize mRNA expression.

Table 1: Gene-specific markers and primers used for real time quantitative PCR.

Gene	Forward primer	Reverse primer
GAPDH	5'TGACGACATCAAGAAGGTGGTG	5'- GAAGGTGGAGGAGTGGGTGTC
ACTB	5'- CTGGAACGGTGAAGGTGACA	5'- CGGCCACATTGCAGAACTTT
IL-4	5'- GCCATCCTGCTCTGCCTTC	5'-TCCGTGGAGTTCTTCCTTGC
IL-12	5'-TATGTTGTAGAGGTGGACTG	5'-TTGTGGCAGGTGTATTGG
iNOS	5'-CGACGGCACCATCAGAGG	5'-AGGATCAGAGGCAGCACATC
TNF-α	5'-TTCTCCTTCCTGCTTGTG	5'-CTGAGTGTGAGTGTCTGG
IFN-γ	5'-GCTTAGATGTCGTGAATGG	5'-GCTGCTGTTGAAGAAGTTAG
IL-10	5'-TGCCAAACCTTATCAGAAATG	5'-AGTTATCCTTCACCTGTTCC
TGF-β	5'-ACGGAGAAGAACTGCTGTG	5'-GGTTGTGTTGGTTGTAGAGG

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ACTB: beta-actin; IL: interleukin; iNOS: inducible nitric oxide synthase; TNF: tumor necrosis factor, IFN: interferon; TGF: transforming growth factor.

Figure 6: RT-PCR and qPCR procedures to evaluate peptides' mRNA expression and interleukins.



3.5 RESULTS

3.5.1 Peptides selection: in silico and *in vitro* analysis

3.5.1.1 *Leishmania* spp. protein selection

First, the proteins HP1 and HP2 from *L. amazonensis* were compared to the protein HP6 from *L. braziliensis*, all the three proteins correspond to the histone subunit H3. The analysis showed that between the protein HP1 and HP6 the similarity is 84.6% and the identity is 94.6%. For proteins HP2 and HP6 the similarity is 85.4% and the identity is 95.4%. Consequently, the similarity and identity of the protein sequences were analyzed with *L. infantum* histone subunits fractions (Tab. 2). The parameters of similarity and identity were not analyzed for HP3 because the protein sequence of histone subunit fraction H1 were not found for *L. braziliensis* and *L. infantum*.

Table 2: Analysis of the sequence homology between histone subunit fractions from *L. amazonesis* and *L. braziliensis* with *L. infantum* histone subunit fractions.

		L. infantum			
	HSF	**H2B**	**H2A**	**H3**	**H4**
L.amazonensis	**H3.1**			Id: 90.8% Sim: 93.8%	
	H3.2			Id: 91.5% Sim: 94.6%	
L. braziliensis	**H2B**	Id: 49.5% Sim: 53.8%			
	H2A		Id: 78% Sim: 87.9%		
	H3			Id: 82.3% Sim: 92.3%	
	H4				Id: 49.5% Sim: 52.2%

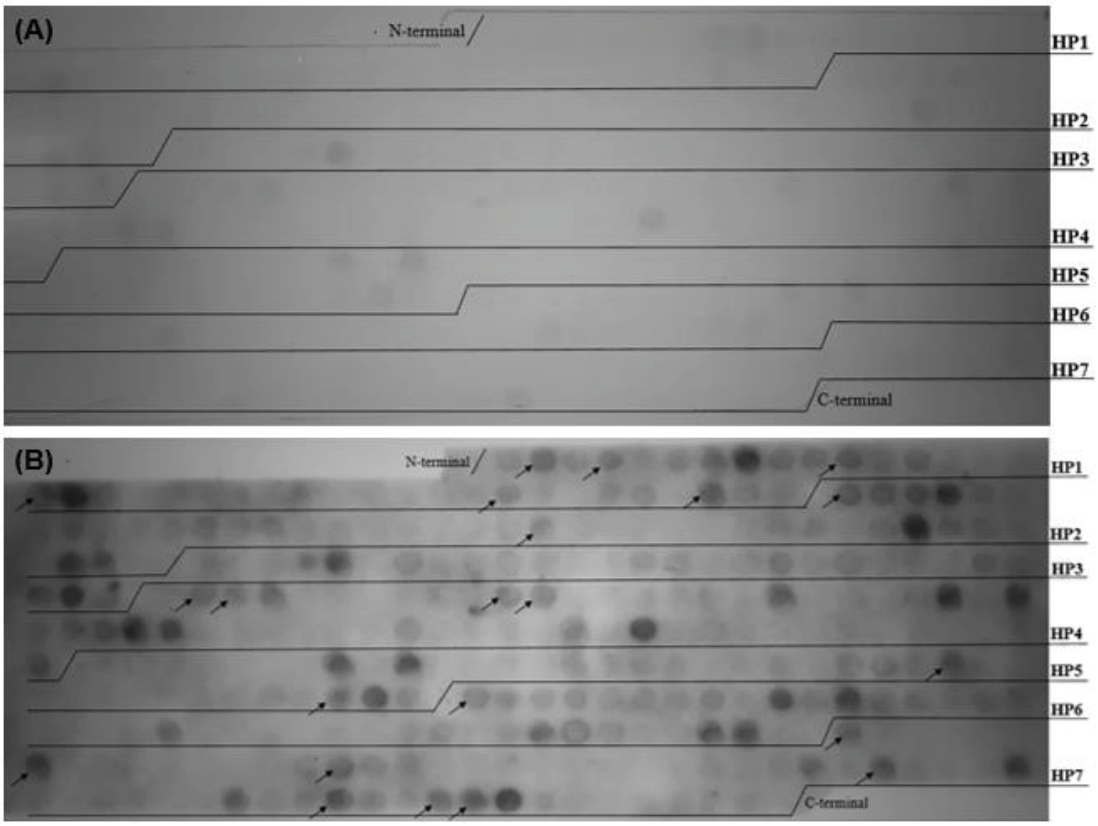
HSF: Histone Subunite Fractions; corresponding to H3, H3.1, H3.2, H2B, H2A, H4.; **H3.1** from *L. amazonensis* corresponds to protein HP1. **H3.2** from *L. amazonensis* corresponds to protein HP2. **H2B** from *L. braziliensis* corresponds to protein HP4. **H2A** from *L. braziliensis* corresponds to protein HP5. **H3** from *L. braziliensis* corresponds to protein HP6. **H4** from *L. braziliensis* corresponds to protein HP7. Id.: Identity; Sim.: Similarity.

3.5.1.2 Peptides immunodetection

The spot synthesis generated 302 spots, being each spot a different pentadecapeptide. The reactive epitopes were immunodetected by a pool of patients' serum with antibodies for *L. braziliensis*. The membrane was tested with serum from volunteer health patients, without antibodies for *Leishmania* spp., in order to verify that these reactions were specific. In this assay was tested the same conditions as one tested for anti-*L. braziliensis* IgG (Fig. 7A).

It was selected 22 spots derived from almost all the seven histone sequences analyzed (Fig. 7B). The 22 spots were named as P_H2, P_H4, P_H11, P_H17, P_H31, P_H37, P_H40, P_H61, P_H109, P_H110, P_H118, P_H119, P_H190, P_H202, P_H205, P_H245, P_H251, P_H260, P_H276, P_H290, P_H293 and P_H294.

Figure 7: Reactivity of peptides originated from histone proteins of *Leishmania* spp. with anti-*L. braziliensis* immunoglobulins G. **(A)** Reactivity of peptides with health patients' serum without anti-*Leishmania* spp. IgG. **(B)** Reactive spots immunodetected by patients' serum with antibodies for *L. braziliensis* and not cross-react with health patients' serum (without anti-*Leishmania* spp. IgG). The arrows indicate the 22 peptides selected by the immunoassay.



3.5.1.3 In silico analysis of peptide sequences

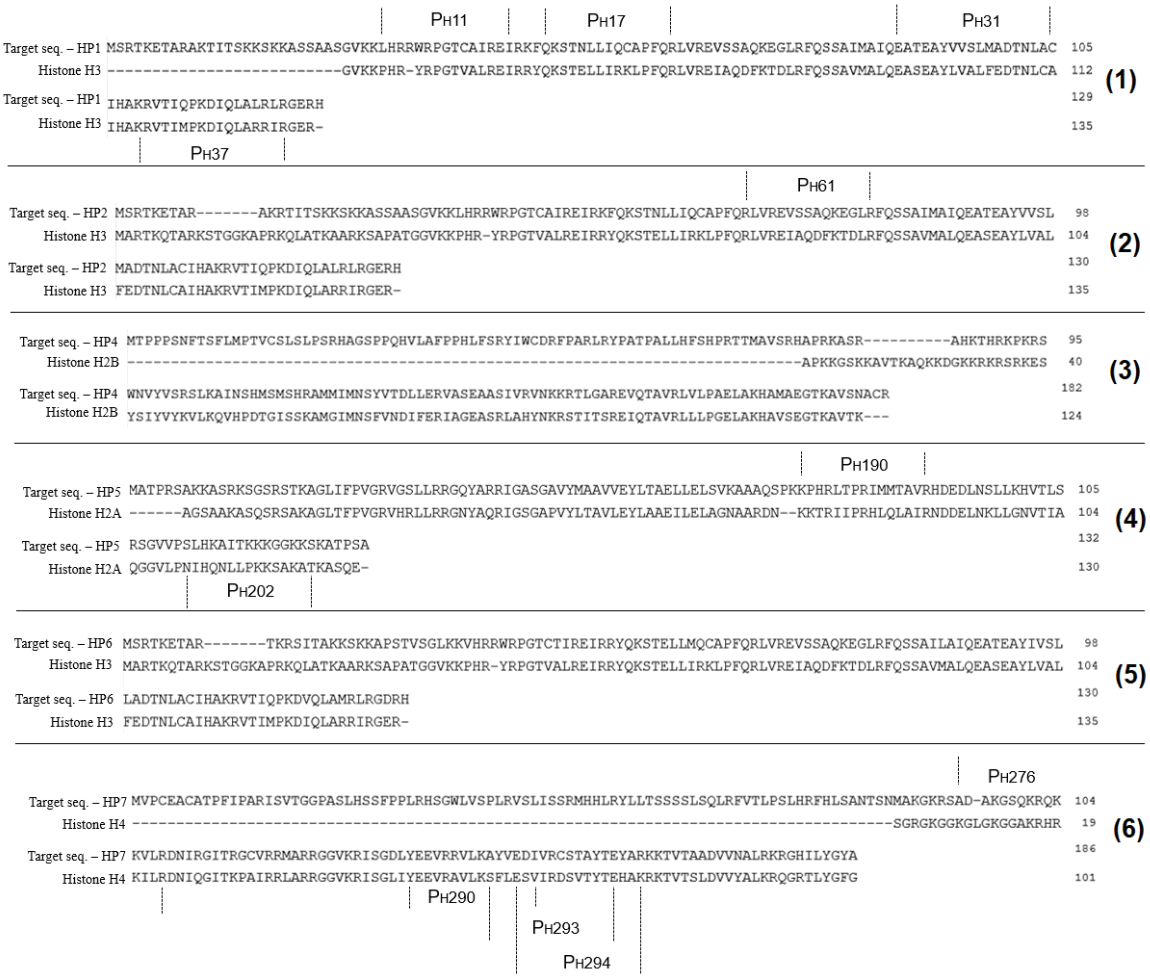
The results of peptides' in silico analysis performed by bioinformatics tools such as Peptide 2.0, IPC, PepCalc, are present in Tab. 3.

Table 3: In silico analysis of reactive peptides. The evaluated parameters are mass weight, iso-eletric point, net charge and hydrophobicity. The (*) indicates the peptides that present hydrophobicity lower than 55 %.

Peptide	Peptide sequence	Molecular weight (g/mol)	Iso-electric point	Net charge	Hydrophobicity (%)
P_H2*	TKETARAKTITSKKS	1649.89	9.66	4	20
P_H4*	AKTITSKKSKKASSA	1535.79	9.76	5	26.67
P_H11*	LHRRWRPGTCAIREI	1864.19	11.37	3	40
P_H17*	QKSTNLLIQCAPFQR	1747.03	9.1	1.9	40
P_H31*	EAYVVSLMADTNLAC	1599.83	3.29	-2.1	53.33
P_H37*	KRVTIQPKDIQLALR	1779.14	10.55	3	46.67
P_H40*	MSRTKETARAKRTIT	1750.04	11.55	4	26.67
P_H61*	RLVREVSSAQKEGLR	1727.96	10.36	2	33.33
P_H109*	PPSNFTSFLMPTVCS	1627.88	5.21	-0.1	53.33
P_H110*	NFTSFLMPTVCSLSL	1659.97	5.21	-0.1	53.33
P_H118	QHVLAFPPHLFSRYI	1825.12	9.95	1.2	60
P_H119	LAFPPHLFSRYIWCD	1865.16	6.59	0	60
P_H190*	KPHRLTPRIMMTAVR	1807.24	12.14	4.1	53.33
P_H202*	SLHKAITKKKGGKKS	1610.94	9.84	6.1	20
P_H205*	MSRTKETARTKRSIT	1766.04	11.55	4	20
P_H245	MVPCEACATPFIPAR	1605.95	5.83	-0.1	66.67
P_H251*	TGGPASLHSSFPPLR	1523.69	10.47	1.1	46.67
P_H260*	LISSRMHHLRYLLTS	1827.16	11.15	2.2	40
P_H276*	ADAKGSQKRQKKVLR	1713	10.66	5	26.67
P_H290*	YEEVRRVLKAYVEDI	1882.12	4.79	-1	40
P_H293*	AYVEDIVRCSTAYTE	1719.87	3.96	-2.1	33.33
P_H294*	EDIVRCSTAYTEYAR	1776.92	4.57	-1.1	26.67

The *Leishmania* spp. histone proteins sequences used in this work were 3D modelled using histones X-ray diffraction structure as templates. The structures of HP1 (71.29 % of identity with the template), HP2 (64.06 %) and HP6 (60.94 %) were building by homology with histone H3. The tridimensional structure HP4 (50.0%) was build with histone H2B; for HP5 (50.41 %) it was used histone H2A; and for HP7 (56.0 %) the template was built by homology with histone H4. The alignments of the sequences and the localization of the reactive peptides are show in Fig. 8.

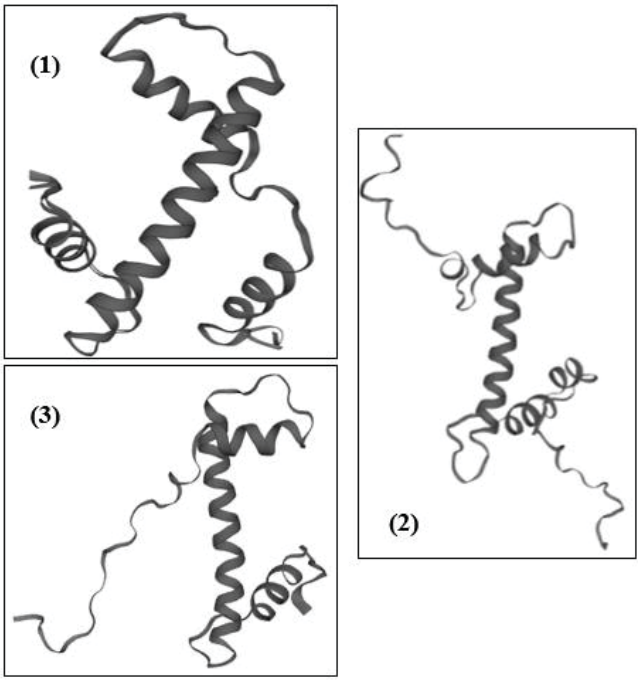
Figure 8: Alignment between *Leishmania* spp. histone proteins sequences and histone from eukaryotic organisms. The localizations of the reactive peptides are show on each sequence.



(1) HP1 protein sequence from *L. amazonensis* and histone H3 (71.29 % of identity); **(2)** HP2 protein sequence from *L. amazonensis* and histone H3 (64.06 %); **(3)** HP4 protein sequence from *L. braziliensis* and histone H2B (50.0 %); **(4)** HP5 protein sequence from *L. braziliensis* and histone H2A (50.41 %); **(5)** HP6 protein sequence from *L. braziliensis* and histone H3 (60.94 %); **(6)** HP7 protein sequence from *L. braziliensis* and histone H4 (56.0 %).

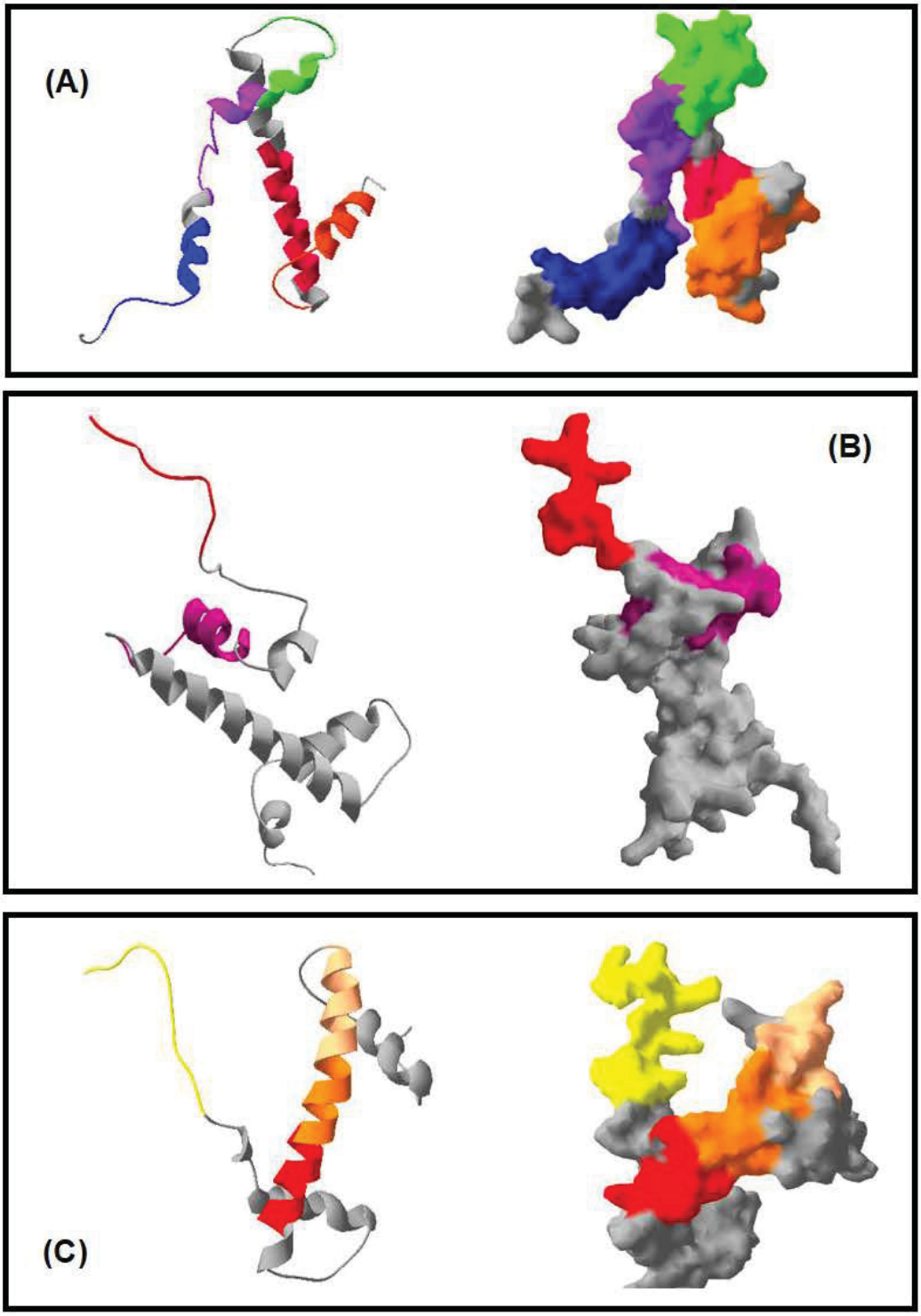
According to these alignments, the tridimensional structures of the proteins HP1, HP2, HP5 and HP7 were built and are presented in Fig. 9. The alignment of HP1 and HP2 sequences was made with the same template from histone H3, so these two proteins have the same 3D structure.

Figure 9: Tridimensional models of *Leishmania* spp. histone proteins. (1) HP1 and HP2 3D model built by homology with histone H3. (2) HP5 3D model using histone H2A as template. (3) HP7 3D model built by homology with histone H4. The analysis was performed using SwissModel program (https://swissmodel.expasy.org/).



After building the tridimensional structures by homology modelling, the peptides were localized on the structures using Swiss-PDB Viewer program. For HP1 the reactive peptides P_H11, P_H17, P_H31, P_H37 and P_H61 are highlight on the histone protein structure present in Fig. 10A. As the same for HP5 with peptides P_H190 and P_H202 (Fig. 10B); and HP7 with peptides P_H276, P_H290, P_H293 and P_H294 (Fig. 10C).

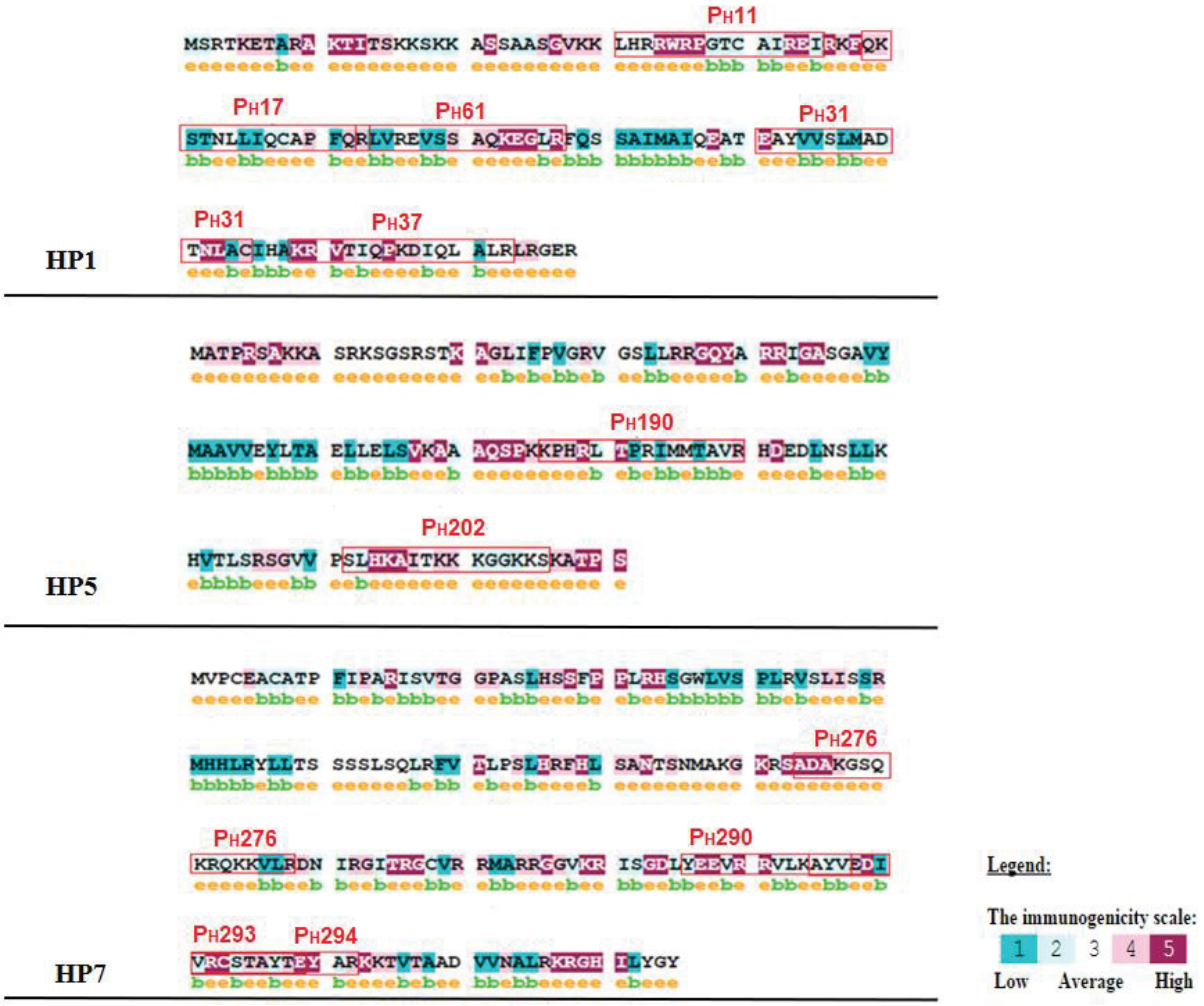
Figure 10: Tridimensional model of *Leishmania* spp. histones and the position of the immunodominant peptides. The colors indicate the different epitope regions of this sequence, which were immunodetected by anti-*L. braziliensis* IgG on the Spot Synthesis membrane.



(A) Ribbon format and solid surface format of the protein HP1. In blue: P_H11; in purple: P_H17; in pink: P_H31; in orange: P_H37 and in green: P_H61. **(B)** Ribbon and solid surface format of the protein HP5. In pink: P_H190; in red: P_H202. **(C)** Ribbon and solid surface format of the protein HP7. In yellow: P_H276; in red: P_H290; in orange: P_H293; in light pink: P_H294.

The reactive peptides were evaluated by Epitopia Server (Fig. 11) and IEDB Analysis Resource (data not show) to verify their immunogenic characteristics.

Figure 11: Immunogenicity analysis of the reactive peptides. The letter e, in yellow and above amino acids, indicates a predicted exposed residue. The letter b, in green and above amino acids, indicates a predicted buried residue. A color scale indicates the immunogenicity of each amino acid, which is present in the figure.



After all these analyses, the aminoacid sequences of the selected peptides were BLASTed against *Leismania* spp. histone proteins sequencen to verify the identity between them (Tab. 4).

Table 4: Identity between peptides sequences and *Leishmania* spp. histone proteins.

Peptide	Histone	*Leishmania* spp. (strain)	Identity (%)
P_H11	H3	*Leishmania mexicana* (MHOM/GT/2001/U1103) *Leishmania braziliensis* (MHOM/BR/75/M2903)	100
P_H17	H3	*Leishmania enriettii* (LEM3045) *Leishmania mexicana* (MHOM/GT/2001/U1103)	93 100
P_H31	H3	*Leishmania infantum* (JPCM5) *Leishmania major* (LV39c5)	100
P_H37	H3	*Leishmania infantum* (JPCM5) *Leishmania major* (LV39c5)	100
P_H61	H3	*Leishmania braziliensis* (MHOM/BR/75/M2903) *Leishmania infantum* (JPCM5)	100
P_H190	H2A	*Leishmania braziliensis* (MHOM/BR/75/M2903)	100
P_H202	H2A	*Leishmania braziliensis* (MHOM/BR/75/M2903)	100
P_H276	H4	*Leishmania braziliensis* (MHOM/BR/75/M2903) *Leishmania infantum* (JPCM5)	100
P_H290	H4	*Leishmania braziliensis* (MHOM/BR/75/M2903) *Leishmania infantum* (JPCM5)	100
P_H293	H4	*Leishmania braziliensis* (MHOM/BR/75/M2903) *Leishmania infantum* (JPCM5)	100
P_H294	H4	*Leishmania braziliensis* (MHOM/BR/75/M2904)	100

According all these analyses the peptides P_H11, P_H17, P_H31, P_H37, P_H61, P_H190, P_H202, P_H276, P_H290, P_H293 and P_H294 were selected and were chemically synthesized.

3.5.1.4 Chemical synthesis of selected peptides and their evaluation by indirect ELISA

The synthesis of peptides resulted in 2.5 mg of each soluble molecule, which were resuspended in 1 ml of ultrapure water. Each peptide was tested as antigen in iELISA assay in concentrations of 0.25 µg/mL, 0.5 µg/mL and 1 µg/mL.

The results showed that all the peptides presented reactivity against the patients' serum even for anti- *L. braziliensis* or for anti- *L. infantum* antibodies.

In the iELISA test three peptides showed 2.5-times diference among positives and negatives patients controls (P_H31, P_H202 and P_H293), (Tab. 5). These three peptides were selected and analyzed in biological assays as antigen candidates for vaccines.

Table 5: Indirect ELISA reaction's parameters standardized for peptides PH31, PH202 and PH293.

	Peptide (µg/mL)[1]	Serum[2]	Second antibody[3]	Absorbance[4]
	Anti – *L. braziliensis* IgG			
P_H31	0.25	1:100	1:7500	0.132
P_H293	0.5	1:200	1:10,000	0.120
	Anti – *L. infantum* IgG			
P_H202	0.5	1:200	1:10,000	0.123
P_H293	0.5	1:200	1:10,000	0.137

[1] Antigen (peptide) concentration on indirect ELISA assay.

[2] Patients' serum concentration.

[3] Second antibody concentration.

[4] The absorbance of negative serum samples was 0.05.

3.5.1.5 Production and evaluation of chimeric molecule

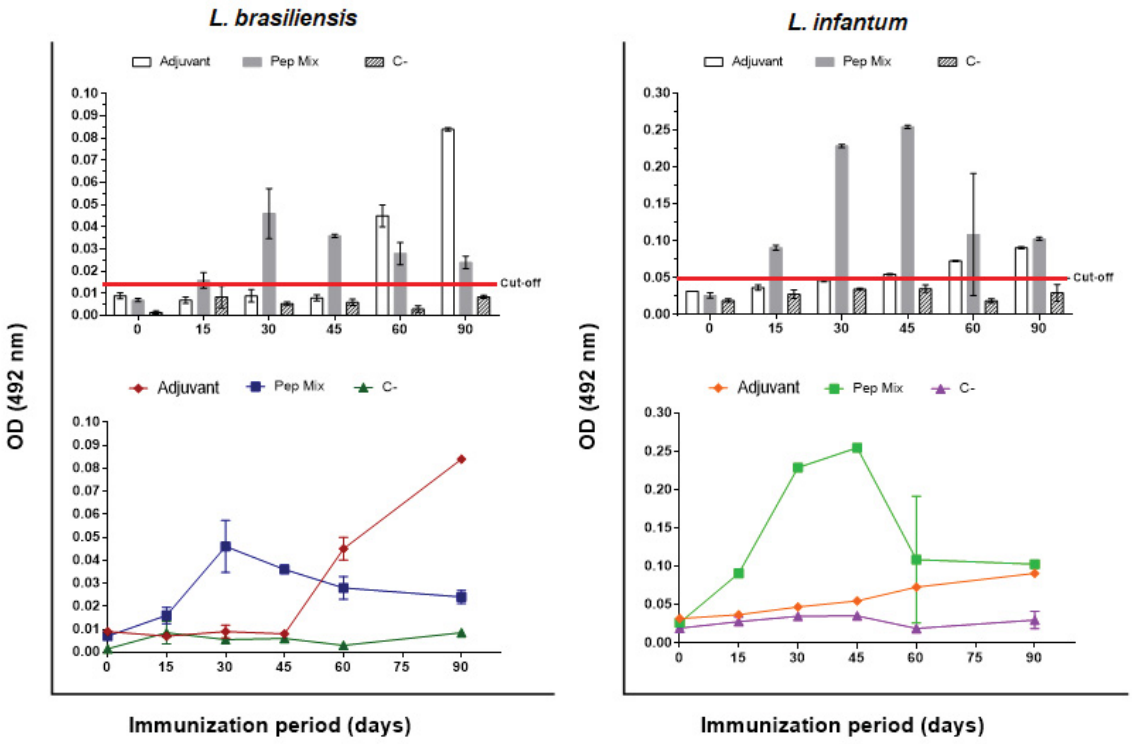
3.5.1.5.1 Preparation of liposome with encapsulated peptide

The encapsulation of peptides resulted in five samples of chimeric molecule in a concentration of 500 µg/mL. To formulate the antigen for animals' immunization, each sample was resuspended in 1 mL of adjuvant resulting in a final concentration of 500 µg/mL.

3.5.1.6 Enzyme-linked immunosorbent assay

The results of IgG profile during immunization period are present in fig 12.

Figure 12: Reactivity of anti- peptides' polyclonal antibodies against *L. braziliensis* and/or *L. infantum* protein extract antigen tested by indirect ELISA assay.

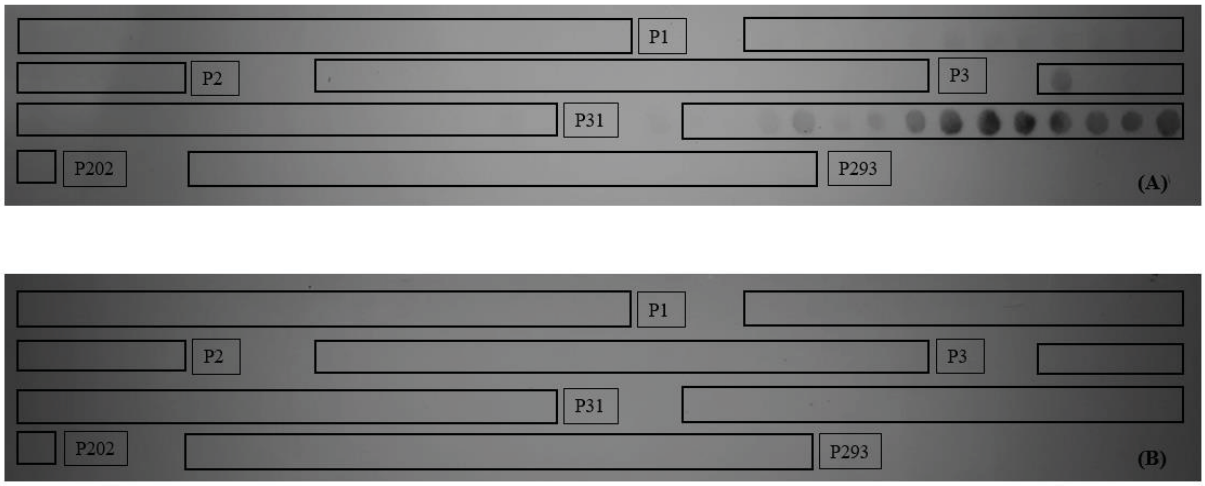


Group 1 = immunized with aluminum hydroxide (adjuvant). Group 2 = immunized with peptides' mix entrapped in liposomes. Group 3 = without immunization. The red line indicates the cut off value 0.014 for *L. braziliensis* and 0.050 *L. infantum*.

3.5.1.7 Confirmation of peptides' antigenic epitopes

The ALA scanning peptides were tested with rabbit anti-peptides serum and rabbit serum (negative control) (Fig. 13). The results showed that for P1, P3 and P_H293 the change of all amino acids from their sequence into alanine decrease their capacity for antibody binding. In the case of P2 the amino acids Glu6, Pro7, Asn8, Pro9 and Leu10 were not fundamental for peptides recognition by anti-peptides polyclonal antibody. The same is observed for Glu1 and Ala2 from P_H31. For $P_H202$the aminoacids Ser1, Leu2, Ala5, Ile6 and Ser15 are crutial residues for the immunogenicity of the peptide.

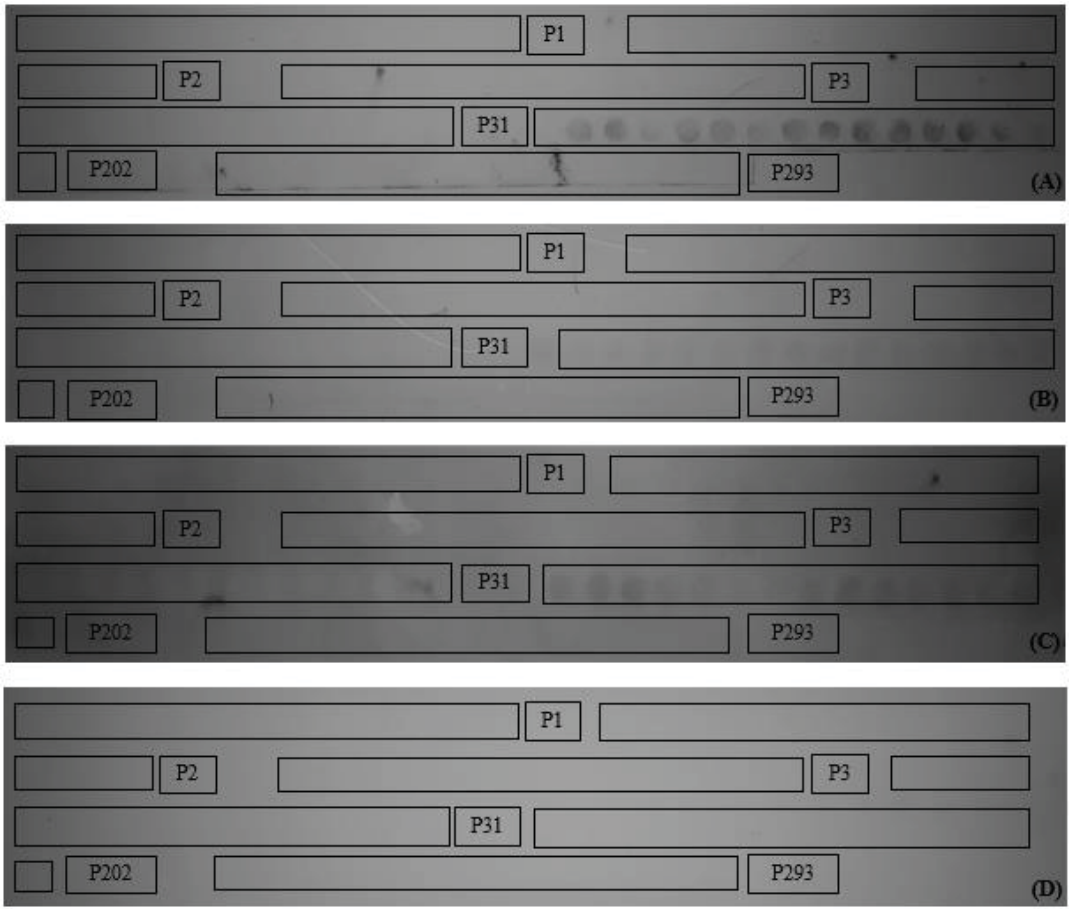
Figure 13: ALA scanning reactive spots. (A) Reactive peptides epitopes for rabbit anti-peptide polyclonal antibodies. (B) Ala scanning peptides tested with serum of rabbits without anti-peptides antibodies (negative control).



Note: The membrane were blocked and tested with rabbits antibodies (diluted 1:100) detected by anti-rabbit IgG (whole molecule) peroxidase antibody (1:10,000). According to standard tests, the exposure time of the membrane to ECL™ system was 15 minutes.

The ALA-Scaning peptides were also immunotested by a pool of patients' serum with anti-*Leishmania braziliensis* (Fig. 14A), anti-*L. infantum* (Fig. 14B), anti-*Trypanosoma cruzi* (Fig. 14C) and anti-*Mycobacterium tuberculosis* (Fig. 14D) antibodies.

Figure 14: ALA-Scan membrane tested by patients' serum with (A) anti-*Leishmania braziliensis* IgG; (B) anti-*L. infantum* IgG; (C) anti-*Trypanosoma cruzi* IgG; (D) anti-*Mycobacterium tuberculosis* IgG.

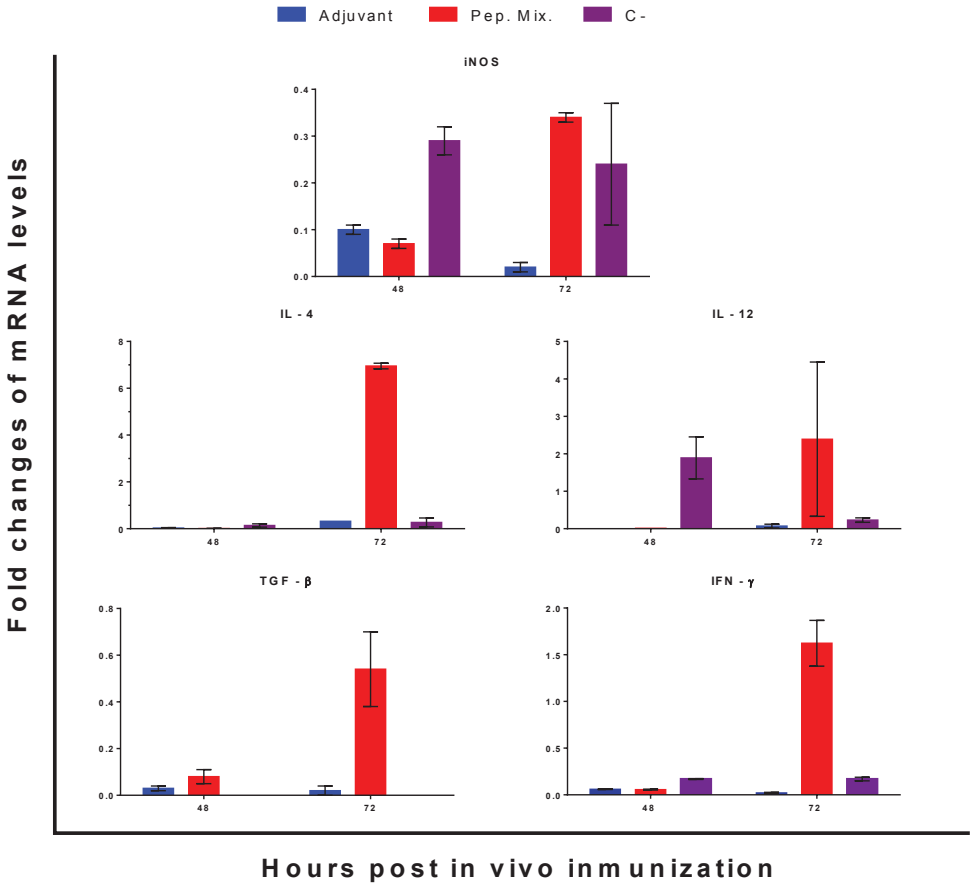


Note: The membrane were blocked and tested with rabbits antibodies (diluted 1:100) detected by biotin-labeled secondary antibody (1: 30,000) and *Streptavidin* (1:10,000). According to standard tests, the exposure time of the membrane to ECL™ system was 15 minutes.

3.5.1.8 Antigenicity and immunogenicity of the chimeric molecule.

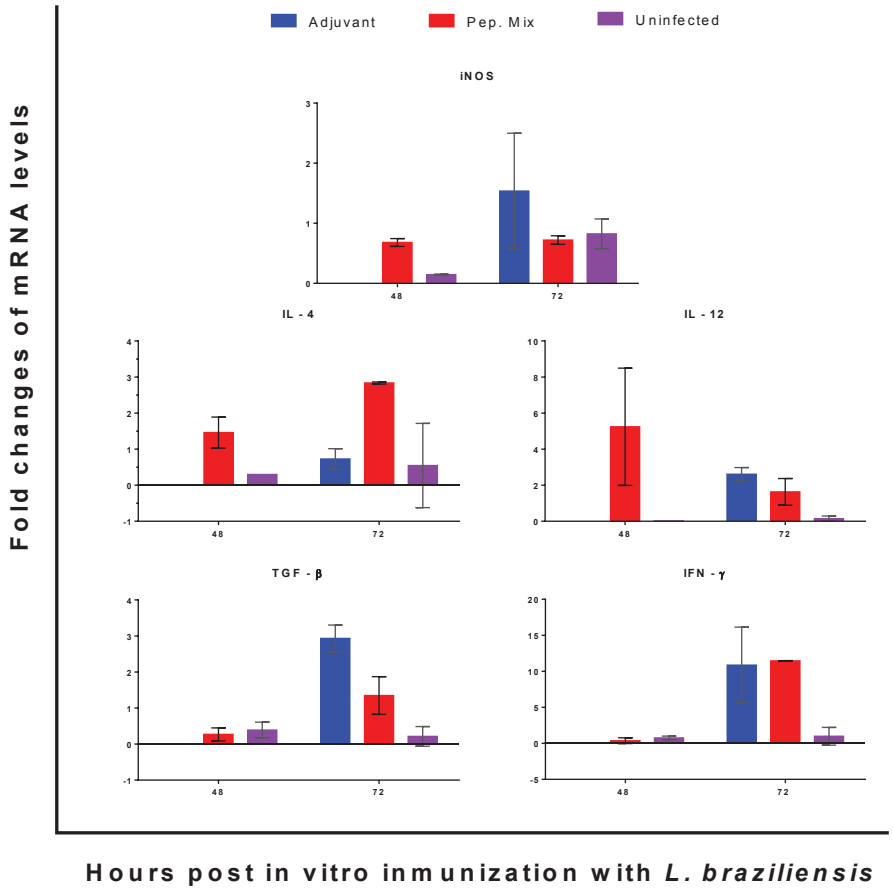
The results showed that the expression of iNOS and interleukins at 48 h was lower than the control group. Although, at 72 h has a 0.34 fold increased iNOS levels of mRNA in relation of the control. For IL-4 a 6.95 fold increased interleukin levels in relation to control. An induced effect of 2.39 fold icrease can be observed for IL-12 and a 0.54 fold for TGF-β in relation to control group. The IFN-γ levels increased 1.6 fold in relation to control group (Fig. 15).

Figure 15: Expression of interleukins and iNOS mRNA from blood samples of animals immunizaded with adjuvant, peptides mix and the control group.



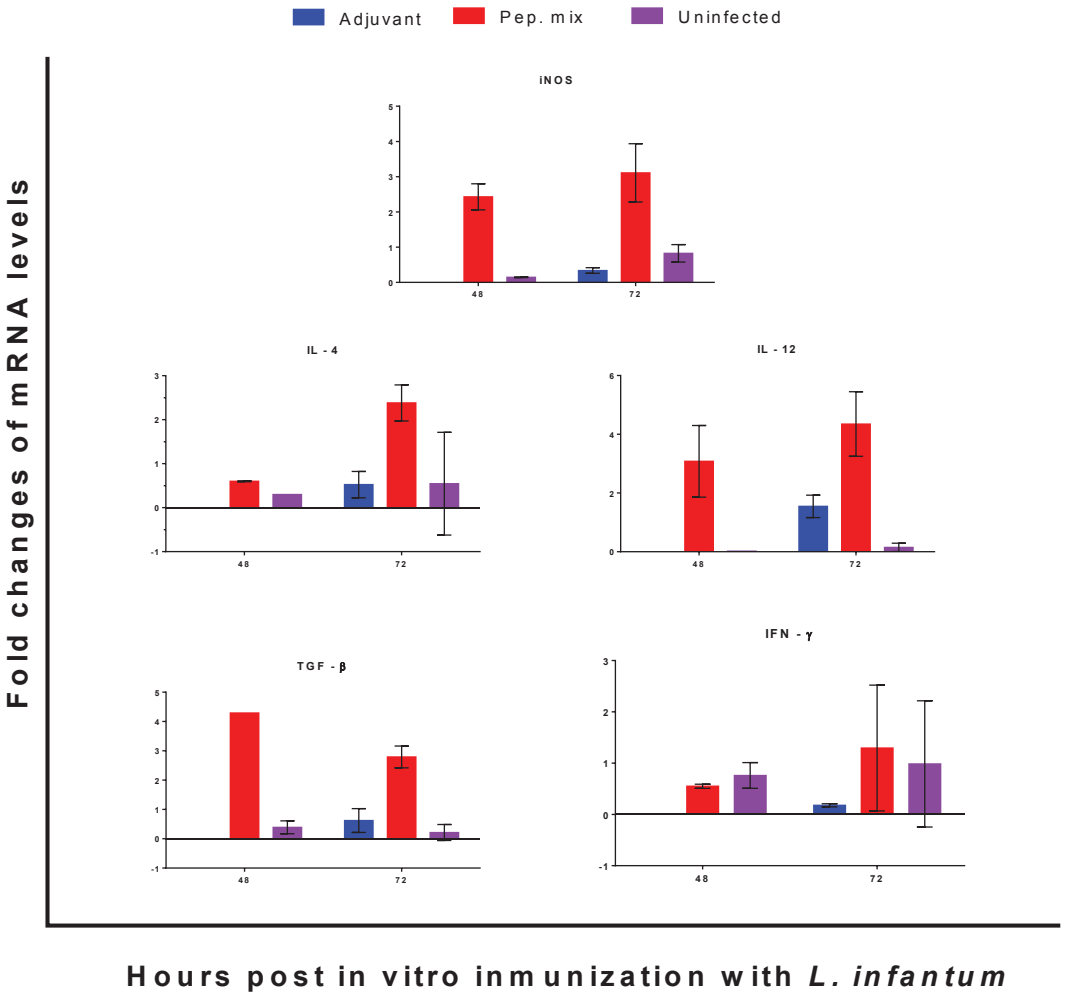
The results of RT-qPCR of macrophages *in vitro* infected cultures with *L. braziliensis* showed that the expression of iNOS at 48 h and at 72 h were lower than the control group (uninfected). For IL-4, an induced effect 1.46 and 2.38 fold increased interleukin levels of mRNA in relation of the control at 48 and 72 h respectively. An induced effect of 5.24 and 1.6 fold increased IL-12 levels in relation to control at 48 and 72 h, respectively. For TGF-β, at 48 h the fold 0.27 was lower than the control group, but at 72 h a 1.34 fold increased in relation to control group. The IFN-γ levels increased 0.27 and 0.95 fold in relation to control group at 48 and 72 h, respectively (Fig. 16).

Figure 16: Expression of interleukins and iNOS mRNA supernatant of the *in vitro* infection macrophages cultures with *L. braziliensis.*



The results of cytokine profile expression of macrophages *in vitro* infected cultures with *L. infantum* showed that the expression levels of iNOS at 48 h and at 72 h increased 2.34 and 3.1 fold, respectively, in relation to control group (uninfected). For IL-4, an induced effect 0.60 and 2.38 fold increased interleukin levels of mRNA in relation of the control at 48 and 72 h respectively. An induced effect of 3.08 and 4.34 fold increased IL-12 levels in relation to control at 48 and 72 h, respectively. For TGF-β, a 4.29 and 2.79 fold increased in relation to control group at 48 and 72 h, respectively. The IFN-γ levels increased 1.81 and 1.94 fold in relation to control group at 48 and 72 h, respectively (Fig. 17).

Figure 17: Expression of interleukins and iNOS mRNA supernatant of the *in vitro* infection macrophages cultures with *L. infantum.*



3.6 DISCUSSION

Leishmania spp. is an intracellular parasite that has complex mechanisms to survive and multiply itself inside host phagocytic mononuclear cells system. The production of antibodies and/or cellular immune during host infection is trigger because most of *Leishmania* antigens are recognize by host system. However, the immune response to some of these antigens does not protect and in other cases may contribute to intensifying the pathological response, due to the cross-reaction with the host's biomolecules (Handman, 2001). Besides that, some studies report that the capacity to respond to *Leishmania* antigens varies from individual to individual, as can be observed in low or higher responders. According to this, a vaccine for leishmaniasis

requires different antigens (polivalent) capable to induce immune protective response to the majority of the population and which can be produced in large scale (Mukherjee et al., 2013).

In order to achieve higher antigenicity, proteins were selected to target antigenic epitopes and after that building a chimeric molecule to have greater antigenicity. In this way, it was selected seven protein sequences of *Leishmania* spp. histone, which are one of intracellular immunogenic molecules from this parasite. The core histone of eukaryotic specie comprises two paired dimers H2A/H2B and H3/H4 and a linker histone H1, which constitute some of the most well conserved molecules among these organisms. For these characteristics, these molecules are good protein candidates to screen conserved immunogenic epitopes among the different *Leishmania* spp. (Baharia et al., 2014). The histone proteins sequences were named as HP1, HP2 and HP3, which derive from *L. amazonensis*; HP4, HP5, HP6 and HP7 from *L. braziliensis*. The proteins HP1, HP2 and HP6 correspond to histone fraction type H3; the protein HP3 to H1; the HP4 to H2B; the HP5 to H2A and the protein HP7 to H4. These sequences were evaluated according to the similarity and identity among them. The results showed that the sequences have more than 84% of similarity and 90% of identity. When compared with *L. infantum* histones the score varies from 52 to 92% in similarity and 49 to 82% in identity. The results indicate a good similarity and identity between the *Leishmania* specie, which is desirable in order to be possible to select epitopes with aminoacid sequences that can be recognized by different specie from the parasite and formulate an antigen capable to induce immune response for different specie *Leishmania*.

In the last years, several studies have described the isolation and application of different biomolecules that present protective activity being good antigen candidates for leishmaniasis vaccine (Singh; Sudar, 2012). These antigens are originated from proteases and other molecules actively secreted by the parasites or from membrane and intracellular proteins. Among these molecules there are the histones, which are highly conserved antigens produced by *Leishmania* spp. and despite they are not secreted by the parasites they can induce a potent immune response. These antigens are release during the infection after the elimination of intracellular amastigotes by the active macrophage, or, by the spontaneous cystolisis of amastigotes inside the infected cells (Carrión et al., 2009). More than that, this antigens are capable of

modulate the host immune response due to the fact of do not suffer with its immune selective pressure, unlike surface and secreted proteins (Chang et al., 2003).

After verifying that there was homology in protein sequences between *Leishmania* spp., the sequences were spot synthesized resulting in 302 spots, each one corresponding to a different pentadecapeptide. In order to verify the reactive of these spots they were immune detected by patients' serum with antibodies for *L. braziliensis*. By this assay, it was possible to immune detect reactive spots on the sequence of the seven histones spot synthesized. More than that, the results confirmed the similarity and identity between *Leishmania* spp. histones once the serum of patients with IgG for *L. braziliensis* recognized and bind to peptides from histone H3 of *L. amazonensis*.

To confirm the reactivity and verify if these reactions were specific, the membrane was tested with serum from volunteer health patients (without antibodies for *Leishmania* spp.). Some reactive spots were immune detected and they were localized on the membrane to verify if they also reacted with IgG for *L. braziliensis*. The spots immune reacted with both IgG for *L. braziliensis* and from healthy patients were excluded from the study because they can be recognize by IgG that is not specific for *Leishmania* spp. By these analyses, it was possible to select 22 spots, originated from histones fraction type H3, H2B, H2A and H4, as reactive to IgG for *L. braziliensis* and not cross-react with health patients' antibodies, and from which ECL™ system exposure result were more expressive (spot's color more intense).

From histone H1 (HP3 on this work) it was not selected any reactive peptide, possibly because most of this histone is composed by a central globular domain flanked by C- and N- termini rich in residues of alanine, lysine, proline and serine that vary in their composition and length among specie, which makes this protein the most divergent histone (Sullivan Jr. et al., 2006; Akiyoshi; Gull, 2013). Thereafter, the IgG for *L. braziliensis* did not sufficiently reacted with the epitopes originated from H1 sequence from *L. amazonensis*.

An in silico analysis, using bioinformatics tools, was performed to evaluate some chemical parameters and protein's structure to verify protein regions that manifest epitope-like characteristics. Among the structure parameters, the hydrophobicity is the most important for peptide synthesis because when the peptide is more hydrophilic better and simple is its chemical synthesis and its application as

antigens for diagnosis and vaccines (Benoiton, 2006). For that, according to the results from the in silico analysis, it was selected the peptides presenting hydrophobicity lower than 55 %, because this characteristic afford the peptide to be more expose in the protein sequence, which means that this peptide is an immunogenic epitope (Sewald; Jakubke, 2015).

In order to analyze the structure of the proteins and to localize the reactive peptides on its sequence, the *Leishmania* spp. histone proteins sequences were 3D modelled. The tridimensional structures of these proteins were obtained by homology modelling using histones X-ray diffraction structure, from eukaryotic organisms, as templates by choosing the structures presenting identity ≥50%.

The tridimensional structure of HP1 protein was built by homology with histone H3 presenting identity rate of 71.29 % between them. The two sequences were aligned to localize the react peptide from HP1 in the sequence of histone H3. The alignment results showed that reactive peptides P_H11, P_H17, P_H31 and P_H37 were localized in H3 sequence. Although, it was verified that peptides P_H2 and P_H4 are in a region of HP1 protein that do not present identity with histone H3 and for this is out of the visible structure region of the protein. Consequently, they were not localized in the sequence and they were excluded from future analysis.

The template for HP2 was built by homology with histone H3 with 64.06 % of identity between them. The alignment results showed that peptide P_H40 was out of the visible structure region of histone H3 excluding it for future analysis. But, the peptide P_H61 was localized on H3 visible region. For HP4 the template was built with histone H2B showing an identity rate of 50 % between the sequences. Although, the alignment results showed that reactive peptides P_H109 and P_H110 were out of the visible structure region of histone H2B and for this, these peptides were exclude. The tridimensional structure of HP5 was built using histone H2A as template showing an identity rate of 50.41 %. The results from alignment between the sequences showed that reactive peptides P_H190 and P_H202 from HP5 were localized on the visible region of histone H2B, and for that both peptides were considered to be analysed on future tests. The template of HP6 was built by homology with histone H3 with 60.94 % of identity rate. However, the reactive peptide P_H205 was out of the visible structure region of histone H3 and was exclude.

The template for HP7 was built by homology with histone H4 presenting 56 % of identity between them. The alignment results showed that P_H276, P_H290, P_H293 and P_H294 were localized on the visible region of histone protein H4. Although, peptides P_H251 and P_H260 were out of the visible structure region of histone H4 and were exclude. According to these in silico analysis' results, it was possible to 3D modeling the protein structure of HP1 and HP2 that have the same structure and derive from the same histone H3; HP5 that derives from histone H2A, and HP7 from H4. It could be observed that all the reactive peptides from these proteins are localized in the external part of the proteins being exposed epitopes for antibody recognition and ligation.

The antigenicity of the core histones (H2B, H2A, H3 and H4) have been report in studies as being recognized by sera from cutaneous (CL) and mucocutaneous (MCL) leishmaniasis human patients and also by canine visceral leishmaniasis (VL) (Soto et al., 1999; Meddeb-Garnaoui et al., 2010; Souza et al., 2013). The protein H2A has being described as the most antigenic core histone and which can also be recognized by sera from VL human patients (Passos et al., 2005). Other studies report the analysis of histones H2A, H3 and H4 immunization against CL applying the histones either individually, in cocktails and genetically fused to a plasmid. The results showed that BALB/c mice genetic immunized with the individual histones resulted in a delay on the development of the lesion, and the immunization with the plasmids encoding the histone and the cocktails provided protection against *L. major* (Carrion et al., 2008). A study of Iborra and collaborates (2004) evaluated the prophylactic activity of *L. infantum* histones in the animal model for cutaneous leishmaniasis, and reported that the animals immunized with a mixture of the four plasmids encoding the histones H2A, H2B, H3 and H4 developed a specific Th1 response associated with a histone-specific production of IFN-γ. Carneiro and collaborates (2012) also analyzed the immune protection conferred by nucleosomal histones of *L. infantum* in murine model infected with *L. braziliensis* , and conclude that histone are potential targets for vaccine formulation against *L. braziliensis* once they showed significant inhibition activity to the disease.

To select the most reactive and antigenic peptides from spot synthesized histones, it was evaluated if the reactive peptides were localized in immunogenic regions of the proteins and if they are able to manifest epitope-like characteristics. The results showed that all the peptides were localized in regions with an immunogenicity

score higher than 70 % (data not show) and majority of the amino acids of the peptides were considered as a predicted exposed residue.

As the last in silico analysis, the peptides sequence were BLASTed against *Leishmania* spp. protein to evaluate the identity rate between them and to analyse if the peptide sequence were homologue to *Leishmania* histones. The results showed that all peptides sequences analysed presented more than 90 % of identity with histone proteins from different *Leishmania* spp., mainly for *L. braziliensis* and *L. infantum*. The results also confirms the identity of each peptide sequence with its own origin histone sequence. All these in silico analysis resulted in the selection of the peptides P_H11, P_H17, P_H31, P_H37, P_H61 (histone H3); P_H190, P_H202 (histone H2A); P_H276, P_H290, P_H293 and P_H294 (histone H4).

The peptides selected by bioinformatics tools were chemically synthesized and anti- peptide histone humoral response were testes by indirect ELISA using human patients' serum with anti- *L. braziliensis* or anti-*L. infantum* antibodies. Different parameters such as concentrations of antigen (peptides) and second antibody, and different dilutions of patients' sera were tested by combining them in distinct ways. The results showed that all the peptides presented reactivity against the patients' serum even for anti- *L. braziliensis* or for anti- *L. infantum* antibodies. Although, when compared with the reactivity of the peptides against health patients' serum (without antibodies for *L. braziliensis* or for *L. infantum* IgGs) some peptides didn't show satisfactory difference. For this, it were selected the peptides in which the reaction's parameters promote a two time higher reactive rate between the peptides tested for anti- *L. braziliensis* or for anti- *L. infantum* and tested for health patients' serum. The peptides selected according these specifications were P_H31, P_H202 and P_H293.

The peptide P_H31 showed good reactivity against anti- *L. baziliensis* antibodies, and P_H202 for *L. infantum* antibodies. The peptide P_H293 showed good reactivity against anti- *L. baziliensis* and for *L. infantum* IgGs.

The milestone of *Leishmania* histones antigenicity studies was the screening of cDNA expression libraries using sera from infected dogs. The first report about a specific immune response against histones during infection was the identification of histone H2A from *L. infantum* by immunoscreening with canine visceral leishmaniasis (VL) serum (Soto et al., 1992). After this, a study from Soto and collaborates (1994) reported the isolation of a cDNA clone coding *L. infantum* histone H3 by a strongly

immunereaction with VL sera. And in a subsequently study the authors demonstrated that the histones H2B and H4 from *L. inafantum* is also recognized by VL sera (Soto et al., 1999). More than that, a study from Lakhal and collaborates (2012) reported the diagnostic performance of a crude *Leishmania* histone used as antigen in an ELISA assay and which reactivity was accessed by sera from VL patients. The results showed the ability of this antigen to discriminate between VL cases and healthy controls.

These studies report the ability and capability of histone proteins on been recognized by humoral immune molecules, which affirms their antigenicity potential to be used as antigens molecules in diagnosis. These studies also corroborate our results for the anti- peptide histone humoral response tests in which was verified the reactivity of the peptides against human patients' serum with anti- *L. braziliensis* or anti- *L. infantum* antibodies. It is a fact that the peptides did not achieve the expected yield as *Leishmania* protein extract or recombinant proteins do. It is because, in comparison to peptides, the protein extracts and recombinant proteins offer a greater variety of reactive epitopes, which consequently can recruit more immune response. This hypothesis reinforces the need of testing different combination of molecules/peptides designed as anti-*Leishmania* vaccines in order to induce a strong immune response (Alonso; Soto, 2014).

Now, we selected three reactive peptides (P_H31, P_H202 and P_H293) and we added three more previously selected by our research group (Thomaz-Soccol et al., 2015; Link et al.; 2017) and were encapsulated in liposome. This small vesicle structure was selected to encapsulate the peptides due to their property of biocompatibility, low toxicity, size and hydrophobic and hydrophilic character. More than that, liposome structures avoid decomposition of the entrapped molecules and release them at designed targets, which make them promising systems for drug delivery (Akbarzadeh et al., 2013; Alavi et al., 2017). The antibody response with the entrapped mix of peptides, evaluated by ELISA test, showed that it was possible to produce anti-peptides' polyclonal antibodies, which have reactivity against *L. braziliensis* and *L. infantum* protein extract antigen. In both situations, it was possible to observe a characteristic curve of antibody response kinetics with plateau phase between 30 and 45 days of immunization. The first 30 days of immunization characterize the primary immune response, where immature B cells are estimulate by

the antigen becaming active and differentiate themselves in antibody secreting cells that produce specific antibodies for the antigen that sitmulate and initiate their development. With repeated infection (45 days), the secondary immune response is induced when the same antigen estimulate the memory B cells leading to the production of greater quantities of specific antibodies than observed in the primary response (Abbas et al., 2015).

The results also show that the group immunized only with adjuvant (aluminum hydroxide) presented immune response, which confirms its ability to be used as an antigen. Aluminum-based adjuvants are widely used in the world, and among the variants, aluminum hydroxide is the most commoly chemical used as adjuvant. An important fuction of aluminum hydroxide is to stimulate T cells activation and the expression of costimulators on antigen-presenting cells (APCs) (Abbas et al., 2015). Aluminum hidroxide mechanisms of action include depot formation, which facilitate the continue release of antigens; formation of particulate structures promoting antigen phagocytes by macrophages and B cells; and inflammation induction that results in activation and recruitment of macrophages (Mutiso et al., 2010; He et al., 2015). In this way, that aluminum-based adjuvants can help to boost humoral immunity response by providing Th2 cell and the injection of this adjuvant can result in priming and persistence of Th2 cells producing IL-4, IL-5 and IL-10 (Awate et al., 2013; Beck et al., 2018).

These results also show that was possible to encapsulate the peptides on the liposomes permitting the use of these molecules to entrap the peptides and delivering them to be recognized as immunogenic epitopes by immune cells and generating immune response against these peptides.

The ALA-Scan assay was performed to evaluate the recognition of peptide epitope by rabbit anti-peptide polyclonal antibodies and identifying the essential amino acids for biding antibody-peptide. The results showed that for P1, P3 and P_H293 the change of amino acids into alanine decrease their capacity for antibody binding. It means that all the amino acids on these peptides sequences are essential as key contributors for the antigenic property of them, in other words they are real epitopes, where the first two (P1 and P3) are conformational once they were selected by phage display, and the last (P_H293) is a linear epitope, obtained by spot synthesis. In the case of P2, P_H31 and P_H202 the transition of some amino acids into alanine show that

they are not essential for the immunogenicity of the peptides. For peptide P2 from 15 amino acids only 11 were essential. The amino acids Glu6, Pro7, Asn8, Pro9 and Leu10 were not fundamental for peptides recognition by anti-peptides polyclonal antibody. The same can be observed for Glu1 and Ala2 from the P_H31. And for the P_H202 the amino acids Ser1, Leu2, Ala5, Ile6 and Ser15 are crucial residues for the immunogenicity of the peptide, so this is the region of the real epitope. The test with ALA-Scan membrane using rabbit serum without anti-peptides antibodies show that these sequences do not present cross reactivity with this serum.

The results for the immunotest with anti- *Leishmania braziliensis* antibodies show that the amino acids from peptides sequences of P1, P2, P3, P_H31 and P_H293 decrease their capacity for antibody binding when changed into an alanine, characterizing them as real epitopes, which will bind to paratope. In the case of peptide P_H202, just the residue Ser15 is essential for the peptide immunogenicity and probably the other neighboring amino acids should be part of the epitope. Once the peptide was sinthesized by spot synthesis the sequence was linear and probably this is a conformational epitope where serine has a fundamental function in immunogenicity. The same results can be observed for the tests with anti-*L.infantum* IgG, although, the intensity of the reaction was not expressive than for anti- *L. braziliensis*.

The peptides sintetized on ALA scanning form were tested against positive serum for *Trypanosoma cruzi* and anti-*Mycobacterium tuberculosis* IgGs to verify the cross reactivity of these peptide epitopes with antibodies generated by immune response similar in action mechanisms to *Leishmania* spp. The results show that when tested with patients' serum with anti-*Trypanosoma cruzi* IgG just the residues of peptide P_H202 demonstrate reactivity with the antibodies when the amino acids were replaced by an alanine, which means that this peptide is not a good candidate for leishmaniasis vaccine once it can cause cross reactivity with Chagas' disease. The other peptides did not present reactivity with these antibodies. For the test with anti-*Mycobacterium tuberculosis* IgGs, any peptide showed reactivity with the antibodies even when the amino acids were replaced by an alanine. These results suggest that some peptides sequences can be modified to avoid cross reactivity with antibodies from other disease and in this way, preventing false positive results in case of using these peptides as antigens for leishmaniasis diagnosis, or preventing false

immunization against leishmaniasis diseases, in case of using them as antigens for vaccines.

All these results from in silico analysis and humoral immune response demonstrated that these peptides are good candidates for leishmaniasis diagnosis once they manifest epitope-like characteristic and satisfactory results when tested with *Leisshmania* patients' serum.

Despite the previous analyses were performed by selecting biomolecules that stimulate immune response type B, some tests were carried out to evaluate the cytokine profile stimulated by these peptides. These evaluation of cellular immune response aimed to verify if the peptides were capable to induce a profile of vytokines that can lead their application as antigens for vaccines. On this way, a protocol of *in vitro* infection was executed to identify the pattern of cytokines synthesized during the immunization period and to verify if there was differentiate production of them, and promote the access to the cellular immune response generated by these peptides. The results showed that the expression of IL4, IL12, TGF-β, IFN-γ and iNOS were higher in animals immunized with peptide mix than in the control group at 72 h post first immunization. It means that the peptide mix was able to induce cellular immune response in animals with a cytokine profile that stimulate T helper 1 (TH_1) and T helper 2 (TH_2) responses.

The expression of iNOS is induced by IFN-γ in macrophages and its activation contributes to control the killing or replication of intracellular pathogens. High- levels of iNOS are associated to the adaptative phase of immune response and its function of co-factor can contributes for the activation of IL-12 and IFN-γ in NK cells (Bogdan, 2015). Transforming growth factor–beta (TGF-β) is known to be multifunctional cytokines that implicate on regulation of a variety biological process by enhancing cellular proliferation, activation and stimulates cytokine of effector Th_1 cells. TGF-β play an important role in immune responses such as induce distinct lineage of pro-inflammatory interleukins (Oh; Li, 2013; Okamura et al., 2015).

The differentiation of Th_1 cells is promote mainly by IL12 and IFN-γ, and occurs in response to pathogens that activate dendritic cells, macrophages and natural killer cells (NK). This profile of cytokines stimulates phagocytosis, oxidative burst and intracellular pathogen killing, regulate the expression of major histocompatibility complex (MHC), class I and class II, and thus stimulate antigen presentation to T cells

(Spelberg; Junior, 2001; Abbas et al., 2015; Cortés et al., 2017). Th_2 differentiation is promoted by IL-4, which stimulate high titers of antibody production and activate B cells proliferation. More than that, Th_2 plays important role on inflammatory process by actvating mast cells and eosinophils (Spelberg; Junior, 2001; Cortés et al., 2017). Both subclasses of $CD4^+$ cells are importante and desired for host defense against different infectious pathogens.

The quantification of interleukins expressed by macrophages *in vitro* infected with *L. braziliensis* or *L. infantum*, showed that the peptide mix was capable to induce a cytokine profile, which presented an increase fold in comparision to the control group for all tested cytokines. Our data also revealed that IL12, IFN-γ and IL-4 levels of mRNA expression by macrophages infected with *L. braziliensis* or *L. infantum*, suggestes the capability of peptides to induce Th_1 and Th_2 protective immune response.

According to the literature, the host immunity to parasite is determined by a suitable Th_1 response characterized by IL12 and IFN-γ production, and induction of iNOS on infected macrophages, which contributes to control parasite proliferation (Costa et al., 2002). Some studies, regarding to the use of biomolecules as vaccine candidates, reported an increase in levels of IL12 and IFN-γ in animals immunized with ribosomal protein and infected with *L. infantum* and *L. amazonensis* (Chávez-Fumagalli et al., 2010). Martins and collaborators (2019) tested the immunogenicity of specific potein from *Leishmania* against *L. major* and *L. braziliensis* infection, and reported that the vaccination induced Th_1 response characterized by the production of IL12 and IFN-γ.

Although, the immunity against *Leishmania* is quite know and define as complex due to the mechanisms used from parasite to survive into host immune system. It is well document that Th_1 response is responsible to induce resistance to leishmaniasis through the production of pro-inflamatory cytokines, such as IL-12 and IFN-γ, leading to the activation of macrophages and killing parasites. At the other hand, the susceptibility to infection is related to Th_2 development and IL-4 cytokine production, which leads to parasite resistance and replication.

The performance of immune response in immunopathology and immunoprotection of leishmaniasis remain a paradoxe. As an example some studies report that, despite of Th_1 response induces a pro-inflamotory cytokines production

and it plays a crutial role for immunoprotection of leishmaniasis, their excessive production can lead to severe immunopathology in the disease (Sacks; Noben-Trauth 2002; Martin; Leibovich, 2005; Nylén; Eidsmo 2012). However, the Th_2 response apart from induce the persistence of the parasite in the site of injection, is able to induce anti-inflamatory cytokines production at lower levels, which can mitigate inflammatory reactions and accelerate the healing process (Nylén; Eidsmo 2012; Pasparakis et al., 2014). These studies suggest that a balance between pro and anti-inflamatory cytokines is assential, and desired, to prevent immunopathological disorders and inflammatory reactions, and can control the infection. This fact reinforce and corroborate our results that the peptide mix was able to induce a cytone profile production that lead to a Th_1 and Th_2 immune response. The next step, it should be evaluated the peptides in *in vivo* model.

3.7 CONCLUSIONS

The focus of this work was grounded based on hypothesis suggested by previous studies of our reseach group with mimetic peptides, and their applicability in different human and animal health field. The search and application of peptides for different purpouses lead us to investigate their ability to be used as vaccine candidates. Previous data suggested this capacity of peptides, although it also suggested that the antigenicity of peptides could be improved by mixing mimetic peptides from distinct parasites' biomolecules. This hypothesis led the investigation of intracellular biomolecules capable of promoting an effective and long-lasting immune response. The in silico investigation of peptides generated by histones showed that these molecules can achieve the purpouse of provide antigenic epitopes, which presented promising biomolecules to be applied in diagnosis.

The mix of mimetic peptides tested on this work, by *in vitro* infection tests, demonstrated a satisfactory development to be used as antigen candidate for leishmaniasis vaccines. The results suggest their ability to induce humoral immune response by producticing antibodies capable to react against *L. braziliensis* and *L. infantum*. They also demonstrate the capacity to generate cellular immune response by inducing Th_1 and Th_2 cytokines, which leads to a balance response by preventing immunopathological disorders and inflammatory reactions.

These results suggest that the peptides are able to mimic parasites proteins that present important role in host-parasite interaction, such as the histones, and being targets to generate immunity against the parasites.

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4 FINAL CONSIDERATIONS

The *in vivo* evaluation of peptides mix in animal model, suitable for *Leishmania* infection, is an essential process to certify the results and hypothesis raised on this work. This assay is need to better understand the peptides mechanisms of actions. More than that, other in silico analyses are necessary to better understand peptides characteristics as epitopes to stimulate immune response type T.

Likewise, experiments regarding to antigen formulation in terms to evaluate different combinations between by using suitable quantity of each antigen to result in the highest immunological response.

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ATTACHMENTS

ATTACHMENT 1: ARTICLE PUBLISHED

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Major Article

New strategy to improve quality control of Montenegro skin test at the production level

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Abstract

Introduction: The production of the Montenegro antigen for skin test poses difficulties regarding quality control. Here, we propose that certain animal models reproducing a similar immune response to humans may be used in the quality control of Montenegro antigen production. **Methods:** Fifteen *Cavia porcellus* (guinea pigs) were immunized with *Leishmania amazonensis* or *Leishmania braziliensis*, and, after 30 days, they were skin tested with standard Montenegro antigen. To validate *C. porcellus* as an animal model for skin tests, eighteen *Mesocricetus auratus* (hamsters) were infected with *L. amazonensis* or *L. braziliensis*, and, after 45 days, they were skin tested with standard Montenegro antigen. **Results:** *Cavia porcellus* immunized with *L. amazonensis* or *L. braziliensis*, and hamsters infected with the same species presented induration reactions when skin tested with standard Montenegro antigen 48-72h after the test. **Conclusions:** The comparison between immunization methods and immune response from the two animal species validated *C. porcellus* as a good model for Montenegro skin test, and the model showed strong potential as an *in vivo* model in the quality control of the production of Montenegro antigen.

Keywords: Cutaneous leishmaniasis diagnosis. Montenegro skin test. *Cavia porcellus.*

INTRODUCTION

Cutaneous leishmaniasis (CL) is a serious social and public health problem, because it can result in sequels[1]. Early diagnosis and a suitable treatment are the best tools to control the disease[1]. Immunological methods have been largely used as a screening tool for diagnosis[1,2]. One method widely used in Brazil is the Montenegro skin test (MST) based on the delayed-type hypersensitivity reaction (DTH)[2]. The *in vivo* manifestation of cellular immune response is an induration that can be measured and semi-quantified by skin tests[3]. The method has been widely used as a complementary leishmaniasis diagnosis because of its high sensitivity and specificity. Furthermore, MST is an easy method to perform, low cost, does not require sophisticated equipment, and can be performed *in loco*[4].

The Montenegro antigen available in Brazil is provided by the *Centro de Produção e Pesquisa de Imunobiológicos* (CPPI) in Paraná State, Southern Brazil. The production is authorized and inspected by the *Agência Nacional de Vigilância Sanitária* (ANVISA) that establishes the standard evaluation of internal testing for antigen production (RDC 59/2000; RDC 167/2004)[5-7]. During the production process, methods for the qualitative and quantitative control of the produced antigens are necessary to evaluate the antigen efficiency and to validate the lots of antigen[5-7]. Currently, this analysis is performed in *in vivo* systems that are exposed to the antigen in order to evaluate the biological response to exposure[5-7]. This control is performed in CL human patients[5]. Such approach demands clinical cases of CL and poses ethical questions, making the quality control of antigen a complicated process. This study aimed to address these issues in order to establish an experimental model capable to replace the current *in vivo* model in the quality control process of Montenegro antigen production.

METHODS

This study was divided in two stages. To evaluate the immune response of *Cavia porcellus* to standard Montenegro antigen in order to establish this species as an experimental model capable of replacing the current *in vivo* process used in the quality control of Montenegro antigen production. Secondly, to validate *C. porcellus* as a suitable animal model, *Mesocricetus auratus*, which is considered a susceptible bio model for infection with *Leishmania* sp.[8,9], was chosen.

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PROTOZOOLOGY - ORIGINAL PAPER

Biological evaluation of mimetic peptides as active molecules for a new and simple skin test in an animal model

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Abstract
A skin test is a widely used tool in diagnostic evaluations to investigate cutaneous leishmaniases (CL). The actual antigen (Montenegro skin test [MST] antigen) presents some difficulties that pertain to its manufacturing and validation. To contribute to overcoming this problem, we propose the application of new-generation molecules that are based on skin antigen tests. These antigens were obtained through biotechnology pathways by manufacturing synthetic mimetic peptides. Three peptides, which were selected by phage display, were tested as skin test antigens in an animal model (*Cavia porcellus*) that was immunized with *Leishmania amazonensis* or *Leishmania braziliensis*. The peptide antigens, individually (PA1, PA2, PA3) or in a mix (PAMix), promoted induration reactions at 48 and 72 h after the test was performed. The indurations varied from 0.5 to 0.7 cm. In the animals immunized with *L. amazonensis*, the PA3 antigen showed better results than the standard MST antigen. In animals immunized with *L. braziliensis*, two peptide antigens (PA2 and PAMix) promoted induration reactions for a longer period of time than the standard MST antigen. These results validate our hypothesis that peptides could be used as antigens in skin tests and may replace the current antigen for CL diagnosis.

Keywords Phage display · Synthetic peptides · Diagnostic · Neglected disease

Introduction

In recent years, the development of biomolecules has attracted the attention of research groups that aim to produce biotechnological supplies. These biomolecules could be applied as sources of pharmaceuticals or even used for diagnoses (Brigido and Maranhão 2002; Bazan et al. 2012; de Moura et al. 2016). As the primary and final product of genetic information pathways, proteins are the most abundant biomolecules in cells and are essential for the biological process. The use of a full-length protein as an antigen can provide multiple epitopes throughout the sequence for antibody recognition and binding. However, these molecules enhance the probability of nonspecific cross-reactivity with homologous epitopes from other proteins (Hancock and O'Reilly 2005; Nagill and Kaur 2011). Once the immunogenic sites of proteins from pathogens are encoded by a small, specific sequence of amino acids (epitopes), these peptides can represent an alternative to recombinant proteins, thereby avoiding the limitations of nonspecific cross-reactivity.

The peptide sequence that triggers the immune response can be identified and chemically synthesized for application as an immunogen in vaccines and within diagnostic approaches (WHO 1997; Brigido and Maranhão 2002; Alban et al. 2014). Synthetic peptides represent well-defined epitopes with highly conserved, unique regions and active sites that can raise the specificity of the antibody ligation (WHO 1997). In a research related to new vaccines and diagnosis markers, synthetic peptides represent a promising approach for developing antigens with high specificity and sensitivity. Some studies have already described the ability of phage-displayed peptides to induce protection against bacteria,

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