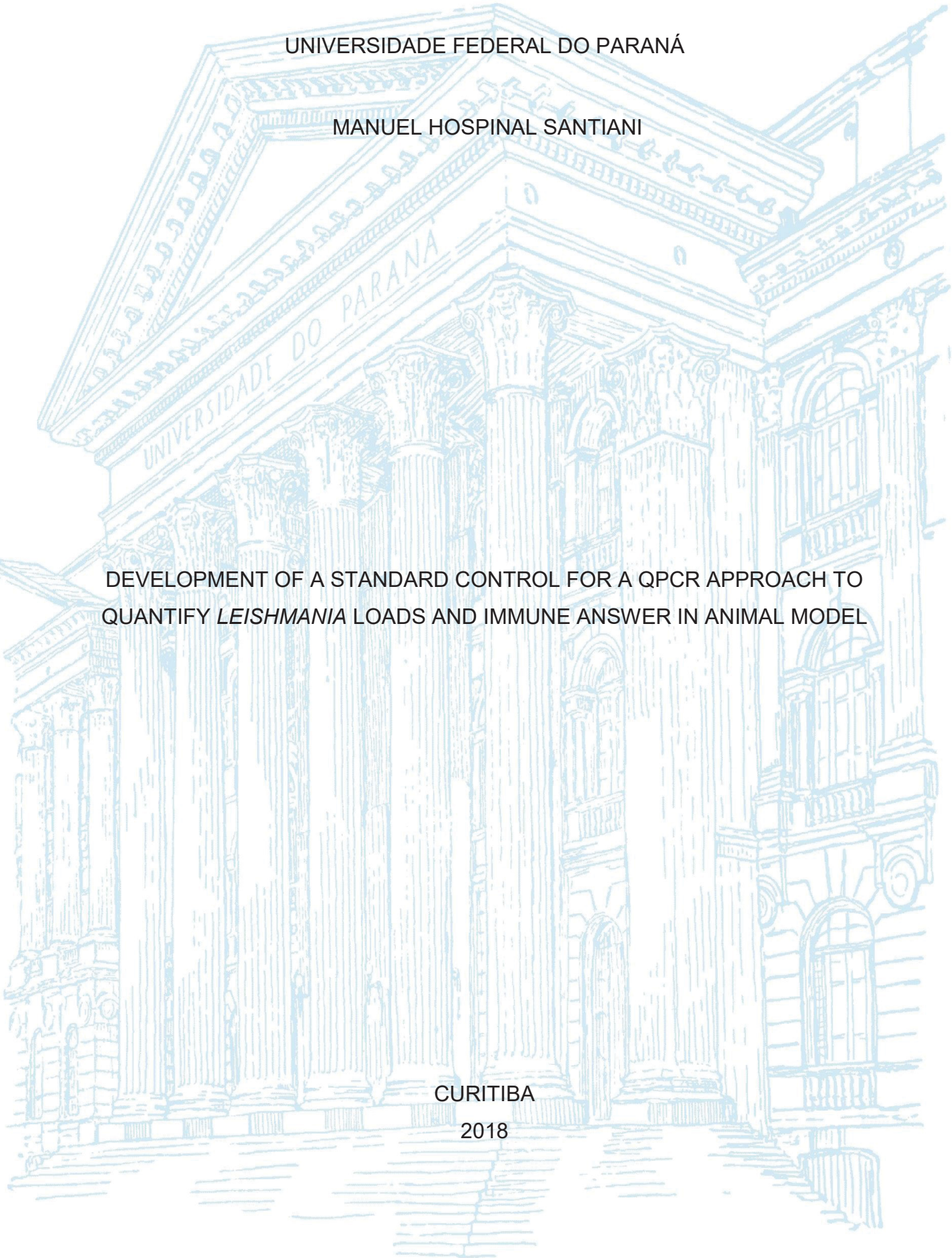
UNIVERSIDADE FEDERAL DO PARANÁ

MANUEL HOSPINAL SANTIANI



DEVELOPMENT OF A STANDARD CONTROL FOR A QPCR APPROACH TO QUANTIFY *LEISHMANIA* LOADS AND IMMUNE ANSWER IN ANIMAL MODEL

CURITIBA

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

Orientadora: Profa. Dra. Vanete Thomaz Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **MANUEL HOSPINAL SANTIANI** intitulada: **"Development of a standard control for a qPCR approach to quantify Leishmania loads and immune answer in animal model"**., após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 25 de Setembro de 2018.

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RESUMO

A leishmaniose tegumentar é causada por um parasito intracelular obrigatório do gênero *Leishmania*, que afeta milhões de pessoas no mundo a cada ano. A falta de metodologia precisa para realizar a quantificação tem sido um obstáculo para medir a carga parasitária no tecido para diagnóstico ou para avaliar a eficácia do tratamento ou para avaliar possíveis candidatos a vacina. A reação em cadeia da polimerase em tempo real (qPCR) é um método alternativo, mais sensível que as técnicas parasitológicas convencionais. O objetivo deste estudo foi desenvolver ferramentas sensível e reprodutível para quantificação de carga parasitária em tecidos com base na tecnologia qPCR. Para realizar o objetivo na primeira etapa, selecionamos genes para quantificar a carga parasitária e, em seguida, desenvolvemos um padrão para quantificação da concentração entre diferentes espécies de *Leishmania*. Essas ferramentas foram avaliadas em ensaios intra-laboratoriais, a sensibilidade foi determinada em 0,01 parasitos/µL e o método foi reprodutível com 100% de concordância entre os participantes. Os resultados mostraram que a especificidade do método identificou o gênero *Leishmania* e não apresentou reação cruzada com *Trypanosoma cruzi* ou DNA humano. Além disso, usamos esses parâmetros para avaliar um produto candidato à vacina desenvolvido pelo nosso grupo. A quantificação do parasito e a avaliação da resposta imune demonstraram que a mistura de peptídeos (P-1, P-2 e P-3) foi capaz de fornecer proteção de 77,8% para hamsters infectados experimentalmente com *L. braziliensis*. A eficácia foi suportada pela diminuição da carga parasitária no baço, pelo aumento do mRNA das citocinas do tipo Th1 (IFN-γ e IL-12) e pela regulação negativa da IL-

10. Corroborando tais dados, houve um aumento no nível de IgG2a nos animais vacinados mostrando que o produto testado estimula o tipo de resposta Th1 e confere considerável proteção contra a leishmaniose cutânea experimental. Em conclusão, as ferramentas que desenvolvemos apresentaram uma alta eficiência para medir a carga parasitária em modelo animal, confirmando que elas podem ser usadas como ferramentas para diagnosticar e monitorar a infecção no processo de desenvolvimento de vacinas.

Palavras-chave: 1. Real Time PCR, 2. Carga parasitária, 3. *Leishmania*, 4.Interleucinas

ABSTRACT

Cutaneous leishmaniasis is caused by an obligate intracellular parasite of the genus *Leishmania* Ross, 1903 which affects million people worldwide each year. The lack of accurate methodology for quantification has been an obstacle to measure parasite load in tissue for diagnosis or to evaluate the efficacy of a vaccine. The real-time polymerase chain reaction (qPCR) is an alternative method, more sensitive than conventional parasitological techniques. The aims of this study developed tools to investigate a sensitive and reproducible method for parasite load quantification in tissues based on qPCR. To accomplish the objectives in the first step we selected genes to quantify the parasite load and then, we developed a standard for quantification the concentration between different *Leishmania* species. These tools were evaluated in intra-laboratory assays, the sensitivity was determined as 0.01 parasites/μL and the method is reproducibility with 100% of concordance between the participants. The results showed that the specificity of the method recognized the genus *Leishmania* and didn't show cross-reaction with *Trypanosoma cruzi* or Human DNA. Additionally, we use these tools to evaluate vaccine candidate product developed by our group. The parasite quantification and immune response evaluation demonstrated that the mix of peptides (P-1, P-2, and P-3) was able to provide considerable protection (77.8%) to hamsters experimentally infected with *L. braziliensis*. The efficacy was supported by the decreased of the parasite load in the spleen, by the increased of mRNA transcript of the Th1-type cytokines (IFN-γ and IL-12), and by downregulation of IL-10. This was further supported by a remarkable increase in IgG2a level. Therefore, it is inferred that the product tested stimulates Th1 response type and confers considerable protection against experimental cutaneous leishmaniasis. In conclusion, the tools here developed presented a high efficiency to measure parasite load in animal model confirming that they can be used as tools to diagnose and monitor the infection in the process of vaccine development.

Keywords: 1. Real-time PCR, 2. parasite load, 3. *Leishmania*, 4. interleukins

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LISTA DE ABREVIATURAS OU SIGLAS

DNA	-Deoxyribonucleic acid
WHO	-World Health Organization
PCR	-Polymerase chain reaction
qPCR	-Real-time polymerase chain reaction
DCs	-Dendritic cells
iNOS	-Nitric oxide synthase
IL-12	-Interleukin 12
IFN-γ	-Interferon gamma
TNF-α	-Tumor necrosis factor alpha
IL-10	-Interleukin 10
TGF-β	-Transforming growth factor beta
HPRT	-Hypoxanthine phosphoribosyltransferase IgG -Immunoglobulin G
OW	-Old World
NW	-New World
CL	-Cutaneous leishmaniases
MCL	-Mucocutaneous Leishmaniasis
VL	-Visceral Leishmaniasis
PKDL	-Post-kala-azar
NNN medium	-Novy, McNeal and Nicolle medium
HIV	-Human immunodeficiency virus
kDNA	-kinetoplast DNA
RNA	-Ribonucleic acid
LAMP	-Loop-mediated isothermal amplification
RFLP	-Restriction fragment length polymorphism
LFA	-Lateral-flow assay
CaCl2	-Calcium chloride
MgCl2	-Magnesium dichloride
BHIB	-Brain–heart infusion broth
RPMI	-Roswell Park Memorial Institute medium
EDTA	-Ethylenediaminetetraacetic acid
OPD	-Ortho-phenylenediamine
SD	-Standard deviation
Lb	-*L. braziliensis*
La	-*L. amazonensis*

LISTA DE SÍMBOLOS

®	-Copyright
µL	-Microliters
™	-Registered trademark
µm	-Micrometers
%	-Percentage
ºC	-Celsius grade
pb	-Base pairs
mg	-Milligram
mM	-Millimolar

SUMÁRIO

1. INTRODUCTION

Leishmaniases are anthropozoonoses caused by the protozoan *Leishmania* Ross, 1903. Cutaneous leishmaniases (CL) are the most common syndrome and causes skin lesson (ulcerates) usually painless and chronic, often occurring at sites of infection. The process of ulcerates formation can take from two weeks to six months or more. The disease is caused by several *Leishmania* species, especially by *L. major* in the Old World, and *L. braziliensis, L. amazonensis, L. guyanensis,* and *L. mexicana,* in the New World (ALVES et al., 2013; REITHINGER; DUJARDIN; LOUZIR, 2007). Several other clinical forms can manifest itself by present depending on the host immune response or the parasite species (BRASIL, 2017). In 2015, the World Health Organization (WHO) reported the presence of leishmaniasis in 200 countries, in which 87 countries were considered endemic for CL. However, over 95% of new CL cases occurred in just six countries (Afghanistan, Algeria, Brazil, Colombia, Iran and the Syrian Arab Republic). Currently, 12 million people are infected, especially in rural areas, and 60 thousand to 1,0 million new cases of leishmaniases has been estimated each year worldwide (STEVERDING, 2017; WORLD HEALTH ORGANIZATION, 2017).

The studies the development of leishmaniasis requires the use of animals model such as mice (BALB/c), guinea pig (*Cavia porcellus*), hamsters (*Mesocricetus auratus*), dogs (Beagle), and other (CRUZ-CHAN et al., 2014; GUEDES et al., 2017). They are used as a reference to simulate and collect data about the evolution of an infection, using this information to screen drug compounds, drug studies, and vaccines against *Leishmania* strains. After experimental infection of the biological model is required the animal euthanasia at different infection time to measure the progression of it (MEARS et al., 2015). The measurement of disease progression is achieved by quantifying the parasitic load which can be done by microscopy, limiting the dilution assay. However, these methods had a range of sensibility between 15 to 80% and take a long time to get results, because require an incubation period of two to four weeks before any visual detection (TORPIANO; PACE, 2015). For this reason, methods that allow obtaining results in short periods of time with highly sensibility and specificity. An alternative is the use of Real-time polymerase chain reaction (qPCR), because results can be obtained after 24 hours and with a sensitivity between 90 to 100% (GALLUZZI et al., 2018). For a sensitive, reproducible and quantification methodology for parasite load in tissue, during experimental infection with *Leishmania* spp. in hamster we proposed two type of markers. The first one was DNA polymerase A in especial the sequence encoding the catalytic subunit, present in a single copy on chromosome 16 (TSOKANA et al., 2014). The second was the marker design for Kinetoplast DNA for a non-protein-coding regions, present in multiplies copies per cell on

Kinetoplast (CECCARELLI et al., 2014). The used of qPCR with one of the markers proposed can be work as a tool for application in the monitoring of experimental treatment, development of vaccines, or diagnostic.

The infection with *L. major* is associated with parasite proliferation and lesion development at the site of injection and has been reported to have spread from the site of infection to internal organs, especially the spleen in various mouse strains (LASKAY et al., 1995). In hamsters can occur the absence of skin lesions, however, with the presence of parasites on internal organs (GOMES et al., 2008). This may be due to the fact that during the experimental subcutaneous infection the promastigotes are introduced into a pool of blood (caused by laceration at inoculation), which can lead them to enter on the bloodstream and migrating to the internal organs. An alternative is an eventual spread to visceral organs that could be linked to the movement of infected cells during the immune response (MCCALL; ZHANG; MATLASHEWSKI, 2013).

Antileishmanial immunity is mediated via both innate (macrophages, neutrophils) and adaptive immunity (B cells, T cells and dendritic cells (DCs)). Macrophages play an important role in both the process of infection and immune response because it has a roll as both the host cells and effector cells that kill the parasites (AWASTHI; KUMAR MATHUR; SAHA, 2004). *Leishmania* sp. phagocytosis by macrophages initiates the Th1-type response with the production of proinflammatories cytokines, such as interleukin 12 (IL-12), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), which is necessary for the leishmanicial activity of macrophages (KATARA et al., 2012; MESSLINGER et al., 2018) as it leads to upregulation of inducible nitric oxide synthase (iNOS) and the recruitment of other pro-inflammatory cells (neutrophils, mast cells, and macrophages). On the other hand, *Leishmania* initiates the induction of macrophage deactivating cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) as well as overshooting production of a Th2 cytokine associated with disease progression (SACKS; ANDERSON, 2004; SHADAB; ALI, 2011; SOARES-SILVA et al., 2016).

Multiple proposals for human vaccine development have been developed, such as live parasites with attenuated, different protein subunits of *Leishmania*, fusion protein, and other (SEYED; TAHERI; RAFATI, 2016). However, currently none of these proposals have been approved for use in humans, and the current treatment is based on chemotherapy with a reduced group of drugs that present serious limitations such as high cost and toxicity, difficult route of administration and low efficacy in endemic areas (EIRAS; KIRKMAN; MURRAY, 2015). In this study, we will focus on the development of a method to quantify the parasitic load and in turn, the use of this technique to evaluate the performance of a product with leishmaniasis vaccine capabilities in an animal model.

2. OBJECTIVE

2.1 GENERAL OBJECTIVE

The aim of this study was to develop a methodology to quantify the parasite load of *Leishmania* spp. by Real-time PCR (qPCR) and use this technique to evaluate the performance of a vaccine candidate product.

2.2 SPECIFIC OBJETIVE

- Select genes for parasite load quantification.
- Develop and evaluate primer sets targeted for genes selection.
- Construct a Plasmid.
- Determine specificity, limit of detection, sensitivity, and reproducibility.
- Validate the methodology.
- Evaluate the methodology with samples from an animal model (Syrian hamster) experimentally infected.
- Evaluate the performance of a vaccine candidate by the production of cytokines and immunoglobulin (IgG total, IgG2a).

3. LITERATURE REVIEW

3.1 ETIOLOGICAL AGENT

Leishmaniases is a complex of infections caused by multiple parasites of *Leishmania* genre (>20 species) that cause diverse clinical manifestations. These parasites are dimorphic protozoans that exist as intracellular amastigotes in mammalian mononuclear phagocytes and as flagellated promastigotes in their vectors, the sand flies (Phlebotominae). The infection is transmitted via the bite of infected female sand flies during the blood meal. The sandflies inject the infective form of the protozoan (promastigotes), which is phagocytized by macrophages and dendritic cells. Inside these cells, the promastigotes are targeted to vacuolar compartments (phagolysosomes) and started to 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 sand fly, and their cells differentiate into promastigotes in the gut of the sand fly.

Currently are described around 98 species sand fly of the genera *Phlebotomus* (42 species from Old World) and *Lutzomyia* (56 species from New World) as proven or suspected vector. Around 70 animal species, including humans, have been found as natural hosts of *Leishmania* parasites (FARRELL, 2002; KIMA, 2007; NADERER e MCCONVILLE, 2008; MAROLI, FELICIANGELI, *et al.*, 2013; WORLD HEALTH ORGANIZATION, 2017).

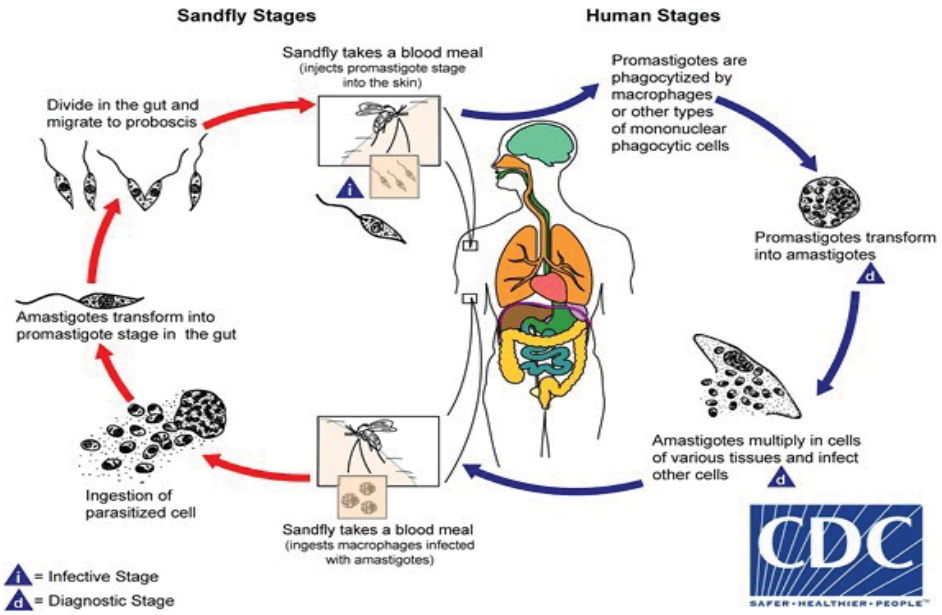

Fig. 1 Parasite cycle, com highlight for as promastigote and amastigote forms, during an infection of sandflies vector and human host. Source: Centers for Disease Control and Prevention, 2016

Ronald Ross firstly proposed the Leishmania genre, which belongs to the Trypanosomatidae family, in 1903 when he described *Leishmania donovani*, named in honor of Charles Donovan and William Boog Leishman. In the last decades, the genus has been

under constant review, and currently is divided in two groups: *EuLeishmania* (composed by the subgenera *Leishmania*, *Viannia, SauroLeishmania and Mundinia*) and P*araLeishmania* (composed by *L. hertigi, L. deanei, L. colombiensis, L. equatorensis, L. herreri,* and *Endotrypanum* species) (SCHÖNIAN, KUHLS and MAURICIO, 2011; AKHOUNDI, KUHLS, *et al.*, 2016; WORLD HEALTH ORGANIZATION, 2017a). For detail, see table 1.

Table 1 Species of *Leishmania* that causes Leishmaniases in humans (adopted and modified according to references AKHOUNDI, *et al.*, 2016; ESPINOSA, *et al.*, 2016).

	Subgenus	**Species**	**Old /New World**	**Reservoir**	**Clinical manifestation**	**Distribution**
Euleishmania	*Leishmania*	*L. aethiopical*	OW	Mammal, Human	CL	East Africa
		L. amazonensis	NW	Mammal, Human	CL, MCL	South America
		L. donovani	OW	Mammal, Human	VL, PKDL	Central Africa, South Asia, Middle east India
		L. infantum (syn. L. chagasi)	OW, NW	Mammal, Human	VL, CL	Mediterranean countries, southeast Europe, Middle East, Central Asia, America.
		L. major	OW	Mammal, Human	CL, MCL	North and Central Africa, Middle East, Central Asia
	Vianni	*L. braziliensis*	NW	Mammal, Human	CL, MCL	Northern South America
		L. guyanensis	NW	Mammal, Human	CL, MCL	Northern South America
		L. panamensis	NW	Mammal, Human	CL, MCL	Central and South America
		L. peruviana	NW	Mammal, Human	CL, MCL	Peru, Bolivia
	Mundinia	*L. enriettii*	NW	Guinea pig	-	Brazil
		L. martiniquensis	NW, OW	Mammal, Human	CL, VL	Martinique, Thailand
	Sauroleishmania	*L. tarentolae*	OW	Lizard	-	North Africa, Malta, Sudan, Italy, France
Paraleishmania		*L. colombiensis*	NW	Mammal, Human	CL, VL	Colombia

Abbreviations: OW - Old World, NW - New World, CL - cutaneous Leishmaniasis, MCL - mucocutaneous Leishmaniasis, VL - visceral Leishmaniasis, PKDL - post-kala-azar.

Leishmaniasis has traditionally been classified according to tree clinical manifestations: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). The MCL results in lesions situated in Naso-oropharyngeal/laryngeal mucosa if untreated and can destroy the tissue. The post-Kala-azar dermal leishmaniasis (PKDL), other clinical manifestation, has also been recovered, that is develop after recovery from an infection of VL (HANDMAN, 2001; ARONSON, HERWALDT, *et al.*, 2016).

The VL form (also known as Kala-Azar) is the most serious and frequently fatal if in not treated, the fatality rate in developing countries can be as high as 100% within 2 years. It is mainly caused by the complex *L. donovani* in East Africa and the India subcontinent and *L. infantum* in Europe, North Africa, and Latin America. After the initial infection, the parasites migrate to internal organs as spleen, liver, intestinal epithelial cells and bone marrow, following an incubation period that generally lasts between 2 and 6 months, resulting in symptoms as persistent systemic infections, anemia, fever, weight loss, spleen and liver augmentation (CHAPPUIS, SUNDAR, *et al.*, 2007; PINHEIRO, PEREIRA, *et al.*, 2008; WORLD HEALTH ORGANIZATION, 2017a).

The CL is the most common syndrome and causes a skin lesson usually painless and chronic, often occurring at sites bite (Fig. 2). The skin lesion (erythema) develops into a papule, then a nodule that progressively ulcerates between two weeks and 6 months to the characteristic lesion of CL: painless rounded open sores with a raised edge and central crater (ulcers). The disease is caused by several *Leishmania* species, especially by *L. major* in the Old World, and *L. braziliensis, L. amazonensis, L. guyanensis*, and *L. mexicana*, in the New World (REITHINGER, DUJARDIN, *et al.*, 2007; ALVES, ALVES, *et al.*, 2013). The CL skin lesions present different characteristics that can be classified in the following groups:

The localized leishmaniasis is the most prevalent form of the CL and is most commonly caused by dermotropic *Leishmania* species, characterized of the lesion is a round, painless ulcer that is well delimited with a central crust that is sometimes hemorrhagic (SCARISBRICK, J., *et al.*, 2006; SCHWARTZ, HATZ e BLUM, 2006).

The disseminated leishmaniasis is characterized by the presence of multiple (10–300) pleomorphic lesions, mainly acneiform and papular, in two or more noncontiguous areas of the body (GOTO e LAULETTA LINDOSO, 2014; ESPINOZA-MORALES, RODRÍGUEZ, *et al.*, 2017).

The diffuse cutaneous leishmaniasis is a rare and severe clinical manifestation of CL, a true anergic form of tegumentary Leishmaniasis and characterized by the presence of nodular lesions that do not ulcerate (BARRAL, COSTA, *et al.*, 1995; HOOJA, SHARMA, *et al.*, 2014).

The leishmaniasis recidiva is characterized by activation of the lesion at the edges after healing of the lesion, the scar-like background remaining. The answer to the therapy is usually lower than that of the primary lesion (CALVOPINA, ARMIJOS, *et al.*, 2006; BRASIL. MINISTÉRIO DA SAÚDE. SECRETARIA DE VIGILÂNCIA EM SAÚDE. DEPARTAMENTO DE VIGILÂNCIA DAS DOENÇAS TRANSMISSÍVEIS, 2017).

The MCL is the severe form of CL. Histological lesions similar to those observed in cutaneous leishmaniasis occur in mucocutaneous tissues, including those in the throat, nose, and mouth, and cause extensive damage and disfiguration. MCL in New World is mostly

caused by *L. braziliensis* in the New World, but other species *(L. amazonensis, L. guyanensis, L. panamensis, L. peruviana*) also can lead to this disease and *L. major* in the Old World. Among patients infected with CL, only 1 to 10% of patient infection progresses to the MCL, and the frequency of MCL vary according to the geographical location. For example, in Brazil, it is estimated from 0.4% in the south, 1.4% in the central region, and 2.7% in the northeast (MACHADO-COELHO, CAIAFFA, *et al.*, 2005; DAVID and CRAFT, 2009; GONZÁLEZ, PINART *et al.*, 2009; GOTO and LAULETTA LINDOSO, 2014).

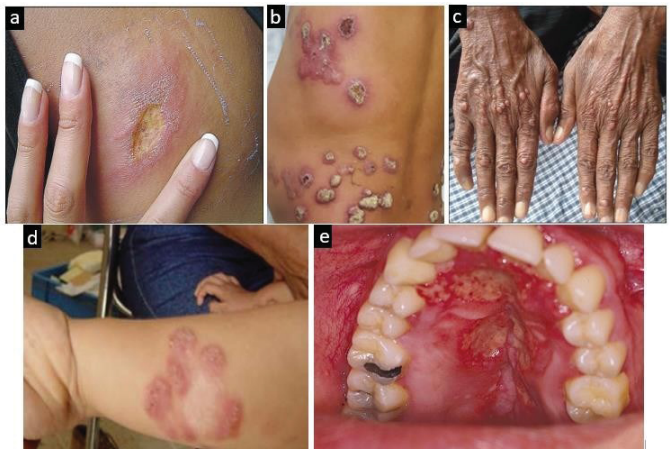

Fig. 2 Clinical forms of cutaneous leishmaniasis and mucocutaneus leishmaniasis: a) Localized leishmaniasis (source: SCHWARTZ, *et al.*, 2006), b) Disseminated leishmaniasis (source: ESPINOZA-MORALES, *et al.*, 2017),c) Diffuse cutaneous leishmaniasis (source: HOOJA, *et al*., 2014) d) leishmaniasis recidiva (source: CALVOPINA,*et al*., 2006), e) mucocutaneus leishmaniasis (source SCHWARTZ, *et al*., 2006).

In 2015, an epidemiological study of leishmaniasis carried out by World Health Organization (WHO) reported the presence of leishmaniasis in 200 countries (Fig. 3), in which 87 countries were considered as endemic for CL, 75 were considered endemic for VL, and 7 were considered endemic for MCL. However, over 90% of VL new case occurred in just 7 countries (Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan), 60% of new CL cases occurred in just 6 countries (Afghanistan, Algeria, Brazil, Colombia, Iran and the Syrian Arab Republic). Currently, 12 million people currently infected, especially in rural areas, and 2 million new cases of leishmaniasis has been estimated each year worldwide: 1.2 to 1.5 million of CL, and 0.2 to 0.5 million of VL (STEVERDING, 2017; WORLD HEALTH ORGANIZATION, 2017; WORLD HEALTH ORGANIZATION, 2017b).

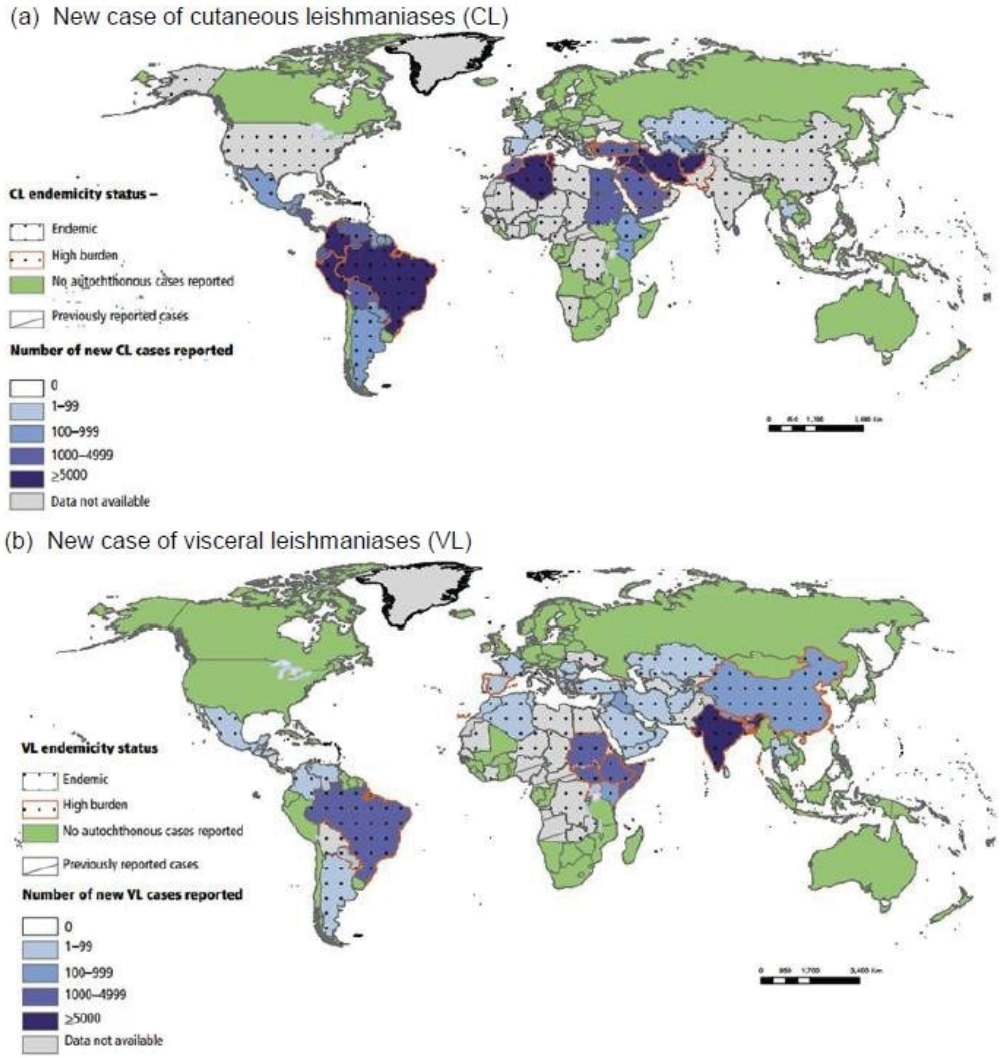

Fig. 3 Geographical distribution of new cutaneous cases of leishmaniasis (a) and visceral leishmaniasis (b) in 2015. Source: World Health Organization, 2017a

3.2 DIAGNOSTIC

The diagnostic of leishmaniasis (VL, CL, and MCL) is big challenges, resulted from the wide spectrum of clinical manifestation, clinical duration and clinical appearance, and the diversity of the parasite. Several methods have been developed and tested to reach a diagnostic solution and obtain precise and accurate. However, the specificity, the sensitivity and reproducibility of methodologies for leishmaniasis diagnosis depend on several factors, including the technical knowledge of the people responsible for perform the tests (staff training), the quality of the equipment and reactive, use of quality controls, intrinsic characteristics of the method, standardization of the sample from patients, the evolution time of the lesions, the clinical forms, and the *Leishmania* species involved in the disease (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2016; BRASIL. MINISTÉRIO A SAÚDE. SECRETARIA DE VIGILÂNCIA EM SAÚDE. DEPARTAMENTO DE VIGILÂNCIA DAS DOENÇAS TRANSMISSÍVEIS, 2017).

Currently, the diagnosis of VL is made combining clinical signs and epidemiological diagnostic with parasitological or serological tests and of CL and MCL are made by epidemiological diagnostic and serological tests. However, these methods present limitations. The used of clinical signs for the diagnoses of CL and MCL has some problem for differentiation diseases, especially those with similar clinical signs. For example, presence of nodules or ulcers that progress for several weeks can be related to: sporotrichosis, *Mycobacterium marinum* infection, venous stasis ulcers, blastomycosis, sarcoidosis, treponemal gummata, Kaposi's sarcoma, leprosy, chromoblastomycosis, squamous cell carcinoma, basal cell carcinoma, B-cell cutaneous lymphoma, seborrheic keratosis, pyoderma gangrenosum, pyogenic skin infections, ecthyma. In the last years, DNA-based methods have been development for corroborate the results of the other test or identified a species (VEGA-LÓPEZ, 2003; WORLD HEALTH ORGANIZATION, 2017a).

3.2.1 SAMPLING

Clinical findings and epidemiological are the first steps for the diagnosis, indicating the possibility of infection by *Leishmania* spp. With this information, the selection of the tissue that should be sampled can be defined. The use of several techniques and the procurement of several specimens per technique are strongly recommended, to continue with the process of discarding or confirming leishmaniasis (SZARGIKI, CASTRO *et al.*, 2009). Patients with suspected of VL should be the following tissues sampled: bone marrow biopsies, blood (with and without anticoagulated), lymph node, biopsy specimens.

For the other hand, patients suspected of CL or MCL the sample is biopsy specimens (~2–4 mm) at the active border of the lesion, tissue impression smears, needle aspirates, dermal scrapings (MATHIS and DEPLAZES, 1995; CDC´S DIVISION OF PARASITIC DISEASES AND MALARIEA, 2016). Although these methods are suggested for CL were the sensitivity of the diagnosis is very varied, depending on the state of the lesson, the method of sampling and the technique used to diagnose, which can vary from 63 to 100% (BONI et al., 2017; MIMORI et al., 2002; SAAB et al., 2015). Biopsy of the injury is the most recommended technique for the diagnosis of CL, despite presenting some issues like invasive, inability to take samples from vulnerable anatomic locations, instrumentation and the high cost because the results are achieved with greater sensitivity in the diagnosis and for VL is bone marrow biopsies.

After the sampling, the next step is to assess the presence of direct or indirect of the parasite. Laboratory diagnosis of leishmaniasis can be made by the following: (i) demonstration of parasite in the clinical sample (parasitological methods); (ii) immunodiagnosis by detection of parasite antigen in clinical sample or by assay for *Leishmania*-specific cell-mediated immunity (immunological methods), or/and (iii) detection of

parasite DNA in tissue samples (molecular methods) (SUNDAR e RAI, 2002).

3.2.2 METHODS FOR DIAGNOSTIC

I. Parasitological methods

Microscopy examination is a method based on searching the amastigotes cells using light microscopy (100x) in the evaluation of slides of clinical samples, using direct microscopy in Giemsa-stained slides under oil immersion, or stained with hematoxylin-eosin paraffin skin specimens. This technique confirms the diagnosis of CL and VL without any indication as regards a species-specific diagnosis (PRINA, ROUX *et al.*, 2007). Moreover, the sensitivity of this methodology is very variable, ranging from 15–40% the Old World and 40-80% in the New World, once the accuracy of microscopic examination is influenced by the expertise of the laboratory technician and the quality of the regents (AL-HUCHEIMI, SULTAN and AL- DHALIMI, 2009; ZAKAI, 2014; TORPIANO and PACE, 2015).

In tissue culture, the samples usually used are a biopsy, aspirate samples, and blood. The culture of the parasite can improve the sensitivity of detection of the parasite, but it is rarely used in routine clinical practice for *Leishmania* sp. because it takes around 1 to 3 weeks for the diagnosis. Alternatively, cultures are usually used in the research with the objective of obtaining antigen for immunologic diagnosis, identifying the species of the parasite, obtaining parasites to be used in animal experimentation, in vitro screening of drugs, and accurate diagnosis of the infection with the organism. The culture media for *Leishmania* is in blood-agar based biphasic media, formerly known as Novy, McNeal and Nicolle medium (NNN medium), or brain heart infusion agar with 10% of blood, overlaid by liver infusion tryptose or Schneider's liquid medium (GOTO e LAULETTA LINDOSO, 2014). The effectivity of these methods for the diagnosis is limited because these methods are difficult to perform in the field, are feasible to the ease of contamination, can be had low sensitivity and the time to obtain results are generally long of 1 to 3 weeks. In addition, in microscopic techniques that requires highly trained personnel to perform the mounting and correct examination of the sheets with the samples. Therefore, the implementation of controls is required by laboratories, an example of these is the examination by two professionals of the mounted sheets in the same way that culture methods.

For these methods, the type and pre-treatment of the samples influence the sensitivity of the methodology used (Table 2). Furthermore, in most studies of the use of these techniques for diagnosis, it appears that controls are not used during the development of the test that could low sensitivity and reproducible methods, or requires

having more professionals working to get a result. For all these reasons requires trained personnel to handling, performing and monitoring of samples in the culture, which leads to increased costs.

Table 2 Comparison of parasitological methods

Method	Sensitivity	Specificity	Leishmaniasis	Control	sampling	Author
Culture methods						
NNN MEDIUM	50.90%	-	VL	-	Lesion scrapes	(POURMOHAMMADI et al., 2010)
SEMISOLID NORMAL RABBIT BLOOD AGAR MEDIUM	62.80%	100.00%	CL	-	Tissue aspirate	(BENSOUSSAN et al., 2006)
NNN MEDIUM BHI	26.70%	-	VL	-	Bone marrow aspirate	(DE GODOY et al., 2016)
NNN MEDIUM	50.00%	100.00%	VL	-	Peripheral blood	(ECHCHAKERY et al., 2018)
NNN MEDIUM	92.90%	-	MCL/CL/VL	-	Biopsy	(GARCIA et al., 2004)
NNN MEDIUM	99.00%	-	VL	-	Splenic aspirate	(MAURYA et al., 2010)
BLOOD AGAR	100.00%	-	VL	-	Splenic aspirate	(MAURYA et al., 2010)
BLOOD AGAR	85.00%	-	VL	-	Buffy coat cells	(MAURYA et al., 2010)
BLOOD AGAR	.00	-	VL	-	Peripheral blood mononuclear cells	(MAURYA et al., 2010)
Microscopy methods						
GIEMSA	76.71%	-	VL	-	Ulcer border	(POURMOHAMMADI et al., 2010)
GIEMSA	20.00%	-	VL	Examined by two parasitologist	Peripheral blood	(OZERDEM et al., 2009)
GIEMSA	74.40%	100.00%	CL	-	Tissue aspirate	(BENSOUSSAN et al., 2006)
LEISHMAN OR ROMANOWSKY DYE	80.00%	-	VL	-	Bone marrow aspirate	(DE GODOY et al., 2016)
MAY- GRÜNWALD GIEMSA	60.00%	100.00%	VL	-	Peripheral blood	(ECHCHAKERY et al., 2018)
GIEMSA	46.20%	95.70%	VL	Examined by two parasitologist	Combined peripheral blood mononuclear cell/buffy coat	(DIRO et al., 2017)
GIEMSA	33.70%	95.70%	VL	Examined by two parasitologist	Peripheral blood mononuclear cell isolation	(DIRO et al., 2017)
GIEMSA	19.60%	98.90%	VL	Examined by two parasitologist	Buffy coat	(DIRO et al., 2017)
GIEMSA	1.50%	100.00%	VL	Examined by two parasitologist	Whole blood	(DIRO et al., 2017)
GIEMSA	74.40%	-	CL	-	Scraping	(HAWASH et al., 2018)
GIEMSA	28.60%	-	MCL/CL/VL	-	Biopsy	(GARCIA et al., 2004)

II. Immunological methods

The techniques are based on the detection of antibodies against the *Leishmania* parasite or on detecting a delayed-type hypersensitivity response using the purified protein of *Leishmania*. In general, these techniques are proposed as a routine diagnostic and shows good diagnostic accuracy in most studies (RODRÍGUEZ-CORTÉS, OJEDA *et al.*, 2010). However, immunological methods have three major drawbacks. First, they are unable to distinguish between current and past infection, because serum antibody levels remain detectable up to several years (SILVA, ROMERO *et al.*, 2006). Second, in endemic areas, up to 24% of healthy individuals with no history of leishmaniasis are positive to immunological methods, possibly due to previous antigen exposure. Third, they have problems in diagnostic in patients with HIV or immunosuppressed patients, possible due lack of antibodies against *Leishmania* (SUNDAR, MAURYA, *et al.*, 2006). Finally, they present potential disadvantage is cross-reactivity with other infections, such as cases of *Trypanosoma cruzi* infection in the New World, and they are not species- specific (CERVANTES-LANDÍN, MARTÍNEZ *et al.*, 2014; MINAYA-GÓMEZ, VARGAS- APAZA *et al.*, 2014). In the table 3 is given the comparison among the immunological methods

Montenegro Skin test. The Montenegro or leishmaniasis skin test, developed by Montenegro in 1926 to diagnose CL (MONTENEGRO, 1926), has been the most utilized method in endemic countries. The test is based on a delayed-type hypersensitivity response using *Leishmania* antigens, from *L. amazonensis* (strain OMS-MHOM/BR73/PH8) in Brazil; that is inoculated intradermally in the forearm of the patient. The presence of clinical reactions in a diameter greater than 5 mm in 48 hours is considered positive.

Although it is a low-cost technique for diagnostic leishmaniasis, other skin infections (sporotrichosis), allergy to the reagent diluent or cross-reactions may result in false positives. Furthermore, positive tests may not be seen before 2 to 4 months after the appearance of the cutaneous lesions, and the preparation of *Leishmania* antigen has not been standardized (DE LIMA BARROS, SCHUBACH, *et al.*, 2005; WORLD HEALTH ORGANIZATION, 2010).

Direct agglutination test. The principle of the direct agglutination test is the agglutination of the antigen from the parasite by antibodies using latex beads that have been previously sensitized with antibodies against *Leishmania* antigen. Direct agglutination test has a high diagnostic accuracy in some geographical areas. However, the results are subject to multiple pipetting, a long and cumbersome incubation time, and

interpretation by the staff, which can introduce a potential variation or error (ADAMS, JACQUET, *et al.*, 2012).

Immunochromatographic strip test. The Immunochromatographic strip test is based on the impregnation of a strip (nitrocellulose) with a *Leishmania*-specific antigen, and its reaction with anti-*Leishmania* antibodies from the patient's serum produces a visible color change. It was developed for being easily performed regardless of the particular level of expertise, rapid, cheap, and good reproducibility in the field (CHAPPUIS, RIJAL *et al.*, 2006).

Currently, *Leishmania*-specific antigens for immunochromatographic strip are in constant development, with different proposes of specific antigen including rK39, rK28, rK16, heat shock protein 70 (*hsp*70), heat shock protein 83 (*hsp*83), glycoprotein 63 (*gp*63), and others (SUNDAR and RAI, 2002; MBUI, WASUNNA, *et al.*, 2013; MUKHTAR, ABDOUN *et al.*, 2015; SIRIPATTANAPIPONG, KATO, *et al.*, 2017). The

most widely utilized *Leishmania* antigen is rK39, a 39-amino acid repeat sequence that is part of the kinesin-related protein of *L. infantum*.

Enzyme-Linked ImmunoSorbent Assay (ELISA). The Enzyme-Linked ImmunoSorbent Assay (ELISA) is an immunoassay technique in which an immobilized *Leishmania* antigen (recombinant or crude) is detected by an antibody bounded by an enzyme capable of generating a detectable product by changing the color. The extension of the change of the color allows quantifying the concentration of anti-*Leishmania* antibodies from the patient's serum. *Leishmania* antigens are similar to those proposed for immunochromatographic strip test, such as kinesin-related proteins (e.g., rK9, rK26, rK39), heat shock proteins (e.g., rHSP70), and other antigens (rlepp12,

L. infantum P0 ribosomal protein) (MOHAPATRA, SINGH, *et al.*, 2010; SRIVIDYA, KULSHRESTHA, *et al.*, 2012). However, the application as a routine technique requires equipment that is poorly adapted to field settings. Must highlight the importance of the ELISA technique, in the set of Immunological methods, because it would be the standard technique for population studies due its rapidity, low cost, and ease of automation. In addition, the readings with this technique are on a continuous scale, with which it is possible to classify in binary form (infected and uninfected) or in more than two categories depending on the approach. With this versatility of the data, it is possible to analyze from ordinal or continuous diagnostic tests can be analyzed even without applying a cut-off value, this is important because the incorrect selection of the cut-off value might severely distort study findings.

Table 3 Comparison among immunological methods for Leishmaniasis diagnosis.

Method	Sensibility	Specificity	Limitation	references
Montenegro Skin test	82 to 100%	90-100%	Not quantitative. Antigen has not been standardized in the world. Need implementation of quality control (QC)	FABER, et al., 2003; REIS. et al.. 2008
Direct agglutination test	40.9 to 85.6%	76.7 to 99.2%	Limited availability of quality controlled Antigen. The high rate of false negatives in special condition (HIV coinfected)	CHAPPUIS, et al., 2006; BOELAERT, et al., 2014
Immunochromatographic strip test	74.5 to 99.5%	76.1 to 97.7%	Not quantitative. Significant regional variation in sensitivity and specificity. A positive result in healthy individuals in endemic regions.	WORLD HEALTH ORGANIZATION, 2011
ELISA	88- 93 %	77-90%	Poor serological response in patients with CL/MCL Need the used of the standard.	SARKARI, et al., 2014; WOLF, et al., 2014

iii. Molecular methods

Molecular methods are based on the detection of parasite DNA in tissue samples using a Polymerase Chain Reaction (PCR) assay. PCR protocols have revolutionized the diagnosis of different disease by providing a sensitive, specificity, reproducibility and rapid method for the diagnosis. In leishmaniasis, this has been tested and demonstrated to be more sensitive than traditional technics, permitting the detection of the parasite prior to the appearance of any clinical symptoms, and also is useful for the diagnosis of VL-HIV co-infected patients. PCR has the possibility of species identification and of assessing the parasite load before and subsequent to antileishmanial treatment for evaluating medication performance (BASTIEN, PROCOP and REISCHL, 2008; SRIVIDYA, KULSHRESTHA *et al.*, 2012). Several targets have been select for determinate the presence of *Leishmania* in the sample, such as ribosomal RNA genes (SRIVASTAVA, MEHROTRA, *et al.*, 2011); kinetoplast DNA (kDNA) (MAURYA, SINGH, *et al.*, 2005), mini exon-derived RNA (med RNA) genes (MARFURT, NIEDERWIESER, *et al.*, 2003), transcribed spacer (ITS) regions (SCHÖNIAN, NASEREDDIN, *et al.*, 2003) Catalytic subunit of DNA polymerase A (WEIRATHER, JERONIMO, *et al.*, 2011). Different approaches have been developed for use PCR, as follows in the table 4 is giving the sensitivity and specificity of the molecular methodologies.

End point PCR. It was the first developed and is based on amplifying a single copy

or a few copies of a segment of DNA (target) across several orders of magnitude in cycles of repeated temperature changes using a thermocycler and DNA polymerase (Taq polymerase). To check the PCR products (amplicons), an agarose gel electrophoresis to visualize and separate amplicon are performed. PCR is a highly specific technique, giving quick results (around of 10 hours depending on the PCR Protocol), and can performed on different samples such as biopsy, blood, smear samples of skin lesions, and archived materials (Giemsa-stained BMAs, formalin-fixed tissue) (SRIVIDYA, KULSHRESTHA, *et al.*, 2012).

In addition, PCR also requires better standardization of the use of reagents, primers, protocols of isolation of DNA, homogeneity of the protocols used in diagnostic centers in order to produce reproducible and truthful results. Some modification of these technic has been proposed, including the PCR-RFLP and nested-PCR. The PCR-RFLP uses restriction enzyme before the electrophoresis, allowing determining a species of *Leishmania* in the sample according to the electrophoresis band pattern (MONTALVO, FRAGA *et al.*, 2010). The nested PCR is similar to a conventional PCR but includes two reaction steps: the first uses general primers and the second used specific primers. These two reactions increase the sensibility of PCR and present a limit of detection up to

0.01 parasites by reaction (OLIVA, SCALONE, *et al.*, 2006).

Loop-mediated isothermal amplification (LAMP). LAMP is an alternative to the PCR methods that not require thermocycles but presents similar high specific and quickness. LAMP uses only one enzyme (Bst DNA polymerase) and is able to amplify large amounts of DNA at a temperature between 60 to 65ºC within 30–60 minutes by the intricate design of primers and auto-strand displacement DNA synthesis. In addition, the reagents involved in the test are stable in room temperature, making LAMP suitable for use in the field (ADAMS, SCHOONE, *et al.*, 2010).

Real-time PCR or qPCR. qPCR is a variation of the PCR, that allows simultaneous monitoring of amplification and presents the possibility of quantification of leishmanial DNA (need a standard), using specific primers (a target that is similar to PCR) with a probe or fluorescent dye. The choice of a target is a very important step. For example, a target with multiplex copies in the genome such as kinetoplast DNA has a high sensibility for the diagnostic of LV, LC, and LMC. But is not able to identify species and has a limitation in the quantification of parasite load, because the number of copies is variable between species. On the other hand, markers with single copy per genome have lower sensitivity, but permit the identification of the species (analysis of curve of melting) and the quantification of the parasite load with precision. Finally, this method is more rapid and less prone to contamination than conventional PCR (BENSOUSSAN,NASEREDDIN, et al., 2006; ANTINORI, CALATTINI, et al., 2009; CECCARELLI, GALLUZZI et al., 2014).

Table 4 Comparison of molecular methods for leishmaniasis diagnosis.

Method	Sensibility	Specificity	Limitation	references
End point PCR	90-100%	95.6%	Requires sophisticated equipment. Better standardization. Quality controls. Invasive sampling.	SRIVIDYA, et al., 2012
PCR-RFLP	90-100%	92-100%	Invasive sampling. Restriction enzymes are expensive. Relatively large amounts of hand- on-time	QUARESMA, et al., 2009; RASMUSSEN, 2012
nested PCR	88- 92%	85-100%	Invasive sampling. Possible carry-over contamination of PCR product.	OLIVA, et al., 2006; FERREIRA, et al., 2014
LAMP	65–94	86–99.9%	Requires less sophisticated equipment than other PCR techniques.	ADAMS, et al., 2012
Real-time PCR	90–100%	90–100%	Need for well-equipped Laboratory. Expensive.	BENSOUSSAN, et al., 2006; ANTINORI, et al.,2009; CECCARELLI, et al., 2014

3.2.3 VALIDATION OF METHODOLOGIES OF DIAGNOSTIC

The validation of the methodologies of diagnostic is a process that determines the fitness of an assay, if it has been properly developed, optimized and standardized, for an intended purpose. During its development, the validation stage determines the reproducibility, inclusivity, exclusivity, accuracy and the limit of detection (LoD) of the method of diagnostic (WORLD ORGANIZATION FOR ANIMAL HEALTH, 2013; WORLD HEALTH ORGANIZATION, 2016). In the development of methods for diagnostic of leishmaniasis, the phase of validation is usually absent, what generate problems in the accuracy and reproducibility; elevate a number of false positive or false negative with the used of the methodology proposed in the center that was developed.

Most of the described methodologies and studies for detection of leishmaniasis have been focused on reporting the results in aspects related to the sensitivity and specificity of the methodology. However, exist other criteria that has importance to the evaluation of a suitable diagnostic, including reproducibility, limit of detection, use or creation of referent material (standards), robustness, interlaboratory comparison and proficiency testing (RODRÍGUEZ-CORTÉS, OJEDA *et al.*, 2010). For example, in the evaluation of Visceral Leishmaniasis, rapid diagnostic test (RDT) has evaluated some of the criteria previously mentioned, and found that four commercially test (Crystal® KA, DiaMed-IT LEISH, Kalazar Detect™ and Signal® – KA) presents variability of the sensitivity across global regions (that was more variable in East Africa and Brazil), while the reproducibility among operators and runs was very good, the diagnostic accuracy of RDTs between participants was high, and also demonstrated a high need to develop a diagnostic algorithm for immunosuppressed individuals (WORLD HEALTH ORGANIZATION, 2011).

Another point is the design of quality control in the systems of diagnostic such as

internal positive or endogenous, negative, inhibition control. This is important to test the validity of a diagnosis, development of new methods, and reproducibility of the methods in different geographic zones and reduces cost in treatment. For example, an endogen control was included in the technique of lateral-flow assay (LFA) to avoid false negatives. The direct assay only with products diluted at 1:250 can be visualized, with the use of endogen control and optical density measurement samples diluted up to a 1:1250 ratio can be detected, ensuring that false negatives are avoided by visualization of the endogenous (RIVAS, ESCOSURA-MUÑIZ *et al.*, 2015).

4. MATERIAL AND METHODS

4.1 MATERIAL

Strains of *Leishmania* and *Trypanosoma* were provided by the bank of strains from Bioprocess Engineering and Biotechnology department of UFPR. The Syrian hamster was provided by ANILAB ANIMAIS DE LABORATÓRIO CRIAÇÃO, the entire reagent for DNA extraction, and qPCR were a molecular grade, DNAse and RNAse free. The conjugate for ELISA (IgG, IgG2a) (BD Biosciences, San Jose, CA, USA), Pierce® high sensitivity NeutrAvidin ®-HPR (Thermo scientific, Erlangen, Germany) o-Phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA).

4.2 SELECTION OF GENE AND PRIMER DESIGN

Known sequences of the two markers for *Leishmania* spp. once for protein-coding genes (DNA polymerase A) and the other no-coding region from Kinetoplast DNA (mitochondrial DNA) were retrieved from the GenBank database (accession numbers: XM_001563712.2, AF231100.1) and the sequences for each of the genes were aligned using the software Clustal X (LARKIN et al., 2007). The alignments were enriched by additional sequencing of more strains from the known sequence of *Leishmania* (Annex 1: Sequence used for the design of primer) and then scanned for regions of high intraspecies sequence conservation that be used for designing primers and probes. Primers were designed using Primer-BLAST software (YE et al., 2012) and synthesized by Macrogen (Macrogen Inc., Seoul, Korea). All primers and probes were designed *de novo* and tested with Blast (Basic Local Alignment Search Tool; NCBI). The WebLogo tool was used to generate a sequence logo based on this alignment (http://weblogo.berkeley.edu/) (CROOKS et al., 2004).

4.3 INSERTION OF POSITIVE CONTROL IN BACTERIAL

A unique standard was constructed for each marker by inserting the test-specific DNA sequence into DNA plasmid (pTOP Blunt V2), which was synthesized by Macrogen (Macrogen Inc., Seoul, Korea) and where generated two plasmids pDNApolyA and pkDNA.

The transformation of *Escherichia coli* BK21 with the plasmids (pDNApolyA and pkDNA), consisted of two steps. The first was $CaCl_2$.$MgCl_2$ method was employed to make competent cells of *E. coli* (SAMBROOK; RUSSELL, 2001). Competent cells were suspended in ice-cold fresh 100 mM $CaCl_2$ solution. The second was the heat shock method with the modification that was employed to insert the plasmids in the bacteria (FROGER; HALL,

2007; SAMBROOK; RUSSELL, 2001). One ng of the plasmid DNA were mixed with 200 µL of freshly prepared competent cells and incubated on ice. After 30 min, heat shock was given for 60 s at 42 °C and instantly transferred on ice for 2 min, followed by addition of 800 µL of Super Optimal Broth with Catabolite repression (SOC). Finally, cells were incubated at 37 °C for 2 h, followed by spreading 100 µL aliquots on nutrient agar plates containing 50 µg/mL ampicillin.

One colony per each plasmid was used to inoculate in Luria Bertani (LB) broth supplement with 50 µg/mL ampicillin and incubated at 37 ºC. After 18 h the plasmid isolation was performed using the manual alkaline lysis method (GREEN; SAMBROOK, 2016), and stored at -20 º C until usage.

4.4 CONSTRUCTION OF STANDARD CURVES FOR PARASITE LOAD

The concentration of the plasmid was measured using a NanoVue™ UV/Visible Spectrophotometers (GE Healthcare), and the corresponding copy number was calculated according to Fu *et al.,* (2009) using the next equation:

$$CN = [(6.02 \times 10^{23} copy/mol) \times DNA\ amount\ (\mathrm{g})]/[DNA\ length\ (bp) \times 660(\mathrm{g}/mol/bp] \qquad eq.1$$

A ten-fold serial dilution of the plasmids (pDNApolyA or pkDNA), were performed starting from 104 to 10-1 copies/µL and was used to construct the standard curves. Threshold cycle (Ct) values in each dilution were measured in duplicate, were plotted against the logarithm of their initial template copy numbers, and were determinate the coefficient of correlation (R2) for each test.

4.5 PARASITES

Promastigotes of the reference strains of *L. braziliensis* (MHOM/BR/1975/M2903), *L. amazonensis* (MOM/BR/1970/BH46) were grown at 24 °C in biphasic media brain–heart infusion broth (BHIB) (Sigma, St. Louis, MO, USA), supplemented with 10% of rabbit blood. *Trypanosoma cruzi* (Y strain) were grown at 27 ºC in RPMI (Sigma) supplemented with 10% inactivated fetal bovine serum (FBS, Sigma).

4.6 DNA EXTRACTION

The extraction started with 50 mg of tissue, which were either homogenized with a pestle and mortar and pass throw syringe with needle and incubated with tissue digestion buffer (containing 100 mM NaCl, 10 mM Tris pH 8.4, 25 mM EDTA, 0.5% SDS and 0.6 mg/mL of proteinase K) at 55 ºC for 12 h. After phenol/chloroform/isoamyl alcohol extraction, DNA was precipitated in the presence of 1/2 volumes of 7.5 M ammonium acetate with 2.5

volumes of absolute ethanol, spooled out, washed twice in 70% ethanol, briefly air-dried, dissolved in 50 µL of 10 mM Tris pH 8.0, 1 mM EDTA and stored at -20 ºC. DNA extraction was performed on 200 µL of culture samples using Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer recommendation, eluted in 50 µL of Elution buffer, and stored at -20 ºC.

4.7 STANDARDIZATION OF QPCR ASSAY FOR PARASITE LOAD

For the standardization of the master mix, a central experimental design was carried out (Table 5. Experimental Design for the reactive of qPCR) using DNA of *L. braziliensis*. The real-time quantitative PCR was performed using StepOne™ Real-Time PCR System (Applied Biosystems). Samples were analyzed in triplicate along with the extraction negative controls and at least three non-template negative controls were included in each plate.

Table 5. Experimental Design for the reactive of qPCR

PtCentral	Block	DNA (nG)	Primer (nM)	Probe (nM)
1	1	50	0.5	0.50
0	1	40	1	0.25
1	1	30	0.5	0.50
1	1	50	1.5	0.50
1	1	30	1.5	0.50
1	1	50	1.5	0.125
1	1	50	0.5	0.125
1	1	30	1.5	0.125
0	1	40	1	0.25
1	1	30	0.5	0.125
0	1	40	1	0.25

4.8 VALIDATION OF METHOD

4.8.1 LIMITED OF DETECTION

In order to compare the sensitivity and linearity of the different markers, a serial dilution of promastigotes parasite was performed (10^4, 10^3, 10^2, 10^1, 10^0 and 10^{-1} parasite/mL) with six replicas.

4.8.2 EVALUATION OF SPECIFICITY

In order to determinate the specificity of the markers, it was performed a template with DNA from *L. braziliensis, L. amazonensis*, *T. cruzi*, human and hamster.

4.8.3 INTRALABORATORY ASSAY

To evaluate the reproducibility of the proposed method, an intra-laboratory test was carried out. The assay was then five independent tested per duplicate of real-time PCR with samples of culture at different concentrations of parasite per mL of blood and samples of DNA. We distributed a panel of samples to five members of the laboratory who performed the test using commercial reagents. The panel included one negative sample of water nuclease-free and one sample of *T. cruzi* (1000 parasites/mL), one sample constructed from purified culture *L. amazonensis or L. braziliensis* (10 or 1000 parasites/mL). One sample of water ultra-pure, one sample of *T. cruzi* DNA and one sample of *L. amazonensis* or*, L. braziliensis* DNA. All samples were diluted in human DNA.

4.8.4 FIELD SAMPLES

To assess the diagnostic test performance, the assay was then validated on skin samples of hamster collected of experimental infection with *Leishmania*. Six samples from animals infected with *L. amazonensis*, six samples from animals infected with *L. braziliensis,* and six samples from animals without infection, collected after 140 days post infection.

4.9 EXPERIMENTAL INFECTION FOR EVALUATION OF CANDIDATE TO VACCINE

Three groups of Syrian hamsters were select for in vivo assay test for a peptide mix (P- 1, P-2, and P-3) candidate to vaccine for cutaneous leishmaniases (data no-showed, for details see LINK *et al.,* 2017).

1) The group was immunized with three doses of the product every 30 days. After the last dose, the animals were infected with 10^6 promastigotes of *L. braziliensis* (immunized group). 2) A group was only infected with 10^6 promastigotes of *L. braziliensis* (infected group), the last group (group 3) consisted of animals without immunization and without infection (uninfected group). The product dose consisted of 60 µg/hamster associated to complete Freund's adjuvant, for the first dose, for the rest of the doses we used incomplete Freund's adjuvant.

After 75 days post infection 50% of the animals were euthanized and the rest after 120 days. Clinical signs and humoral immunity determined by ELISA assay were analyzed. A necropsy was performed to obtain spleen samples for cytokine and parasitic load evaluation by qPCR.

4.10 INTERLEUKINS EVALUATION

Reverse transcriptase Real-time PCR (RT-qPCR) was performed to assess the expression of mRNAs for various cytokines and inducible Nitric oxide synthase (iNOS) in splenic cells.

Total RNA was isolated using RNeasy Mini kit (QIAGEN) in combination with TURBO DNase free kit (Invitrogen) according to the manufacturer recommendation and quantified by spectrophotometers. A total of 400 ng of RNA was used for the synthesis of cDNA using a First Strand cDNA Synthesis (Thermo Scientific) using oligos dT_{15}.

The RT-qPCR was performed as described by Samant et al., (2009) with modification as shown in Table 6**.** The qPCR reaction was carried using the Step One plus (applied biosystem). All quantifications of interleukins were normalized using the housekeeping gene HPRT by $2^{-\Delta\Delta Ct}$ Method (LIVAK; SCHMITTGEN, 2001).

Table 6 Markers, primer sequences and qPCR conditions used for evaluating the expression of cytokines.

MARKER	PRIMER SEQUENCE	QPCR conditions
IL-4	Forward 5'-GCCATCCTGCTCTGCCTTC-3'	2.5 µL of SYBR green PCR master mix
	Reverse 5'-TCCGTGGAGTTCTTCCTTGC-3'	(Applied Biosystem)
IL-12	Forward 5'-TATGTTGTAGAGGTGGACTG-3'	10 pmol of each primer
	Reverse 5'-TTGTGGCAGGTGTATTGG-3'	1 µL of cDNA
iNOS	Forward 5'-CGACGGCACCATCAGAGG-3'	Final volume: 5 µL
	Reverse 5'-AGGATCAGAGGCAGCACATC-3'	Stage 1: 95°C for 10 min
TNF-α	Forward 5'-TTCTCCTTCCTGCTTGTG-3'	Stage 2: 40 cycles, 95°C for 30 s, 50ºC for
	Reverse 5'-CTGAGTGTGAGTGTCTGG-3'	30 s, and 72°C for 30 s per cycle.
HPRT	Forward 5'-GATAGATCCACTCCCATAACTG-3'	2.5 µL of SYBR green PCR master mix
	Reverse 5'-TACCTTCAACAATCAAGACATTC-3'	(Applied Biosystem)
IFN-γ	Forward 5'-GCTTAGATGTCGTGAATGG-3'	20 pmol of each primer
	Reverse 5'-GCTGCTGTTGAAGAAGTTAG-3'	1 µL of cDNA
IL-10	Forward 5'-TGCCAAACCTTATCAGAAATG-3'	Final volume: 5 µL
	Reverse 5'-AGTTATCCTTCACCTGTTCC-3'	Stage 1: 95°C for 10 min
TGF-β	Forward 5'-ACGGAGAAGAACTGCTGTG-3'	Stage 2: 40 cycles, 95°C for 30 s, 52ºC for
	Reverse 5'-GGTTGTGTTGGTTGTAGAGG-3'	30 s, and 72°C for 30 s per cycle.

IL-4 (interleukin 4), IL-12 (interleukin 12), iNOS (inducible nitric oxide synthase), TNF-α (tumor necrosis factor α), HPRT (hypoxanthine phosphoribosyltransferase), IFN-γ (interferon gamma), IL-10 (interleukin 10), and TGF-β (transforming growth factor β).

4.11 EVALUATION OF PARASITE LOAD

The parasite load was carried out using the marker DNApoly A with used of pDNApolyA for make the standard curve, once Ct was converted into an estimate of copy number (Q) per reaction tube, the level of parasitemia (P), expressed as number of *L. braziliensis* per mg of tissue, was calculated according to Ros-Garcia *et al.* (2012), as shown in the following equation:

$$P = Q \times (M) \times (Vel/Vt) \times (1/CN) \qquad eq.2$$

where V is defined as volume in µL, M is defined as mass in mg, and represents the following: Mass of the tissue to refer the results to, 50 mg of spleen (M=50 mg), nucleic acid extraction eluate (V_{el}=50 µL), nucleic acid template added to the PCR reaction (V_T= 2µL); and CN is the gene copy number (1 copy per genome).

4.12 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR IGgTOTAL AND IGg2A

The soluble antigenic preparations of *L. braziliensis* were developed using 1 × 10^6 stationary-phase promastigotes, as described by Castro, Thomaz-Soccol and Augur, (2003). The ELISA approach was carried out (MAZIERO et al., 2014) with some modifications. The sera samples were analyzed in 96-well flat-bottomed microtiter plates (high binding) sensitized with 500 ng/µL of protein diluted in coating buffer pH 9.6 and maintained overnight at 4 °C. After blocking with 2% casein in PBS, sera diluted 1:50 were added to each well and incubated at 37ºC for 1 hour. The plates were then washed three times and incubated with specific Biotin Mouse Anti-Syrian Hamster immunoglobulin (IgG) or Biotin Mouse anti-Syrian Hamster immunoglobulin G2a at 0.5 µg/mL, followed by an incubation step with Neutravidin ®-HPR (Thermo Scientific) at a dilution of 1:8000 for 1 hour at 37ºC. The reaction was revealed using hydrogen peroxide and ortho-phenylenediamine (OPD) and stopped with 20 µL of 2% sulfuric acid per well. Absorbance was determined in a spectrophotometer at 492 nm.

4.13 STATISTICS ANALYSES

Results were presented as means ± SD. For the analysis of the interlaboratory test it was used Fisher's exact test for the concordance between the results; negative deviation, relative specificity, positive deviation, and relative accuracy was verified with a test of proportions. Comparisons of means between groups were performed using the ANOVA with Tukey's HSD. Tests were performed using Graph Pad software (Prism 7 version 7.04), Excel software (office 2016).

4.14 ETHICAL ISSUES

The present study was approved by the Committee on the Ethical Handling of Research Animals of from the Federal University of Parana (CEUA/BIO-UFPR), Curitiba, Parana, Brazil (Process n. 101328/2015-69).

5. RESULT

5.1 PRIMER DESIGN AND QPCR STANDARDIZATION FOR THE *LEISHMANIA*

After the alignment of the sequences, a region with low diversity was selected (Fig. 4) for the marker catalytic subunit of DNA polymerase A (DNApolyA) (DpolyAF 5'-GACGGTGAATTACAGGCTGC-3'; DpolyAR: 5'-ATACTTGCAGCAGCACATCG-3'), were designed to amplify a 150 bp fragment. A TaqMan® hydrolysis probe specific for marker DNApoly A was designed (FAM 5'-TCACTTGCACACCAGATGCA-3' BHQ1); for the marker Kinetoplast DNA (kDNA) (KNPLF 5'- CTTTTCTGGTCCTCCGGGTAGG-3'; KNPLR: 5'-CCACCCGGCCCTATTTTACACCAA-3'), were designed to amplify a 170 bp fragment. A TaqMan® hydrolysis probe specific for DNA polymerase A was designed (FAM 5'-TTTTCGCAGAACGCCCCTACCCGC-3' BHQ1). No matches to other microorganisms or human DNA was observed.

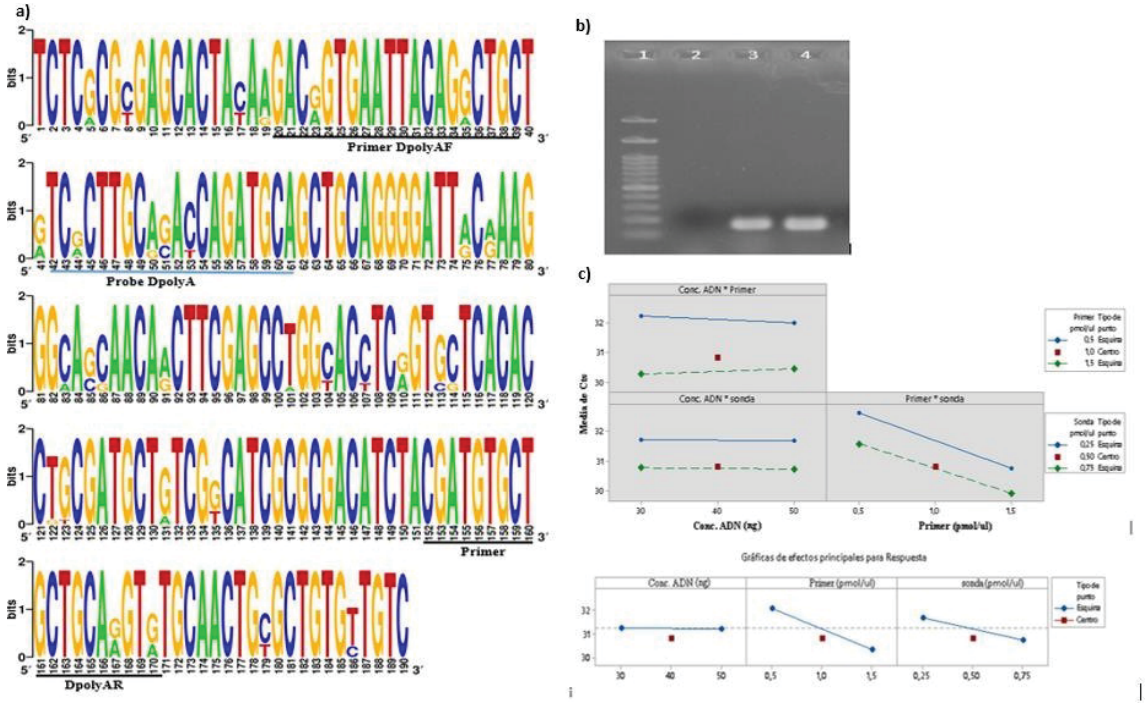

Fig. 4 Primer design and optimization of DNApolyA based RT-PCR for parasite quantification a) Multisequence alignment based on 13 homologous sequences to *L. braziliensi* DNApolyA found using NCBI Blast b) DNApolyA primers amplify a 150bp product specifically. Product visualized with ethidium bromide staining of a 1% agarose gel run. 1). 100 bp DNA ladderKASVI; 2) *T. cruzi*; *3) L. braziliensis*; *4). L. amazonensis*; c) Influence of the tested variables.

Optimized conditions consisted for DNApoly A of 1X TaqMan™ Universal PCR Master Mix (Applied Biosystems). 0.5 nM of each primer, 0.125 nM of probe, 50 ng of the sample DNA to a final volume of 10 µL. PCR cycling conditions were 95°C for 10min and then 40cycles of 95°C for 30s, 56°C for 30 s and 72 for 30 s. and for kDNA of 1x TaqMan™ Universal PCR Master Mix (Applied Biosystems). 0.5 nM of each primer, 0.125 nM of probe, 50 ng of the sample DNA to a final volume of 10 µL. PCR cycling conditions were 95°C for

10 min and then 40 cycles of 95°C for 30 s, 62°C for 30 s and 72 for 30 s.

5.2 REAL-TIME PCR ASSAY: STANDARD CURVES, SPECIFICITY, AND SENSITIVITY

For the kDNA marker, it was possible to amplify all the points of the standard curve using the plasmid (pKDNA). However, for the *L. braziliensis* and *L. amazonensis* samples, the points corresponding to 0.1 copies/μL_{DNA} were not amplified. These results confirm that the detection limit of the cDNA marker was 1 copy/μLDNA (Fig. 5.a). When comparing the C_t value of the last amplified points of the *L. braziliensis* (C_t=37.35 ± 0.82) and *L. amazonensis* (C_t=34.25 ± 0.71) samples, the difference between C_t of the sample (ΔC_t=3.1) which can be translated by performing the transformation using a standard curve as opposed to approximately ten times more the amount of parasites in the sample. Additionally were tested the specificity of the marker with DNA of human and *T. cruzi*, and no amplification was found.

With DNApolyA marker, it was possible to amplify four points of a standard curve using the plasmid (pDNApolyA) with a limit of 10 copies/μL_{DNA} and was similar to what was found using the *L. braziliensis* and *L. amazonensis* samples. These results tell us that the detection limit for DNApolyA marker is 10 copies/μL_{DNA} (Fig 5.b). When comparing the C_t value of the last amplified points of the *L. braziliensis* (C_t=37.64 ± 0.38) and *L. amazonensis* (C_t=36.29 ± 0.62) samples, the difference between C_t of the sample (ΔC_t=1.35) does not generate many variations in the parasitic load. Additionally were tested the specificity of the marker with DNA of human and *T. cruzi*, and no amplification was found.

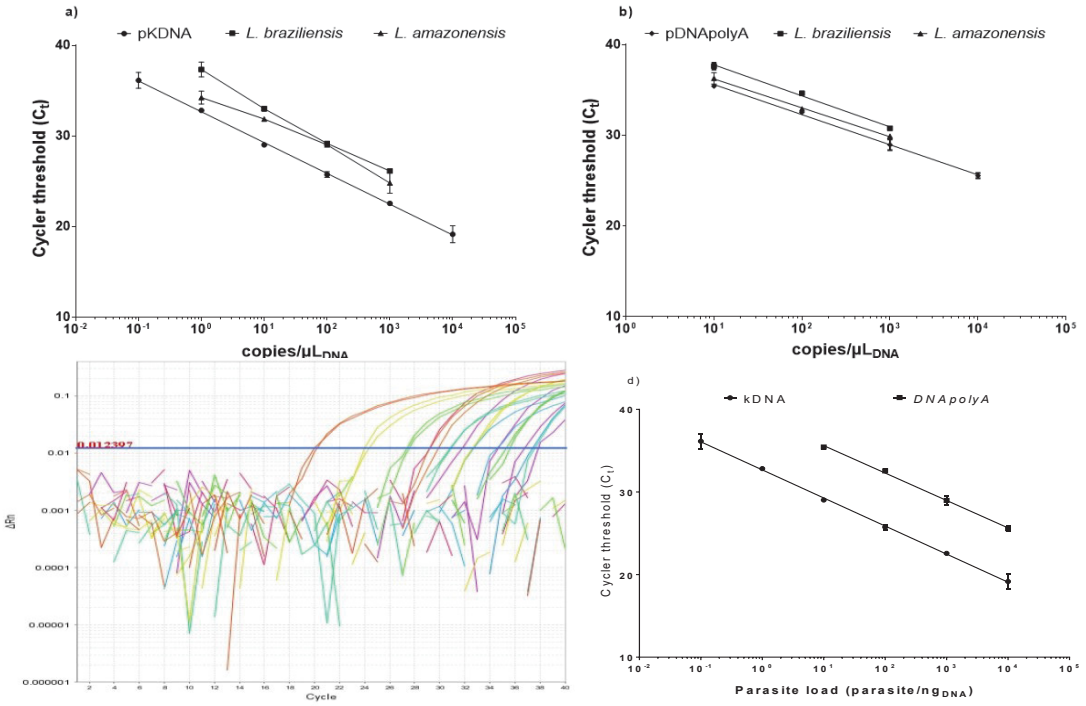

Fig. 5 Standard curve generated with the two markers in comparison to DNA extracted from human blood and spiked with *L. amazonensis* or *L. braziliensis*. (a) Standard curves for the marker kDNA were generated from the linear region of each amplification. Efficiency of amplification for each primer set was determined using the equation: efficiency (E) =1-10(−1/slope), being kDNA E = 96.842% and R^2 = 0.9949; (b) Standard curves for the marker DNApolyA, E = 93.07% and R^2 = 0. 9934. (c) The curve of amplifications that were generated after amplification of DNA with the different markers. (d) Shows the sensitivity of real-time PCR to detect *Leishmania* DNA. The limits of detection were 0.1 and 10 parasite equivalent/50 ng DNA for the marker kDNA and DNApolyA respectability.

5.3 REPRODUCIBILITY OF TEST OF INTRALABORATORY.

The participants reported that they did not obtain amplification in samples 1 and 3 from culture samples, which were *T. cruzi* and water samples respectively. These results indicate that during the process of extraction and preparation of reagents for qPCR there was no cross-contamination and that there were no unspecific amplification. It is supported by the results obtained with DNA samples 1 and 2 which are *T. cruzi* and water samples respectively. For the samples 2 (1000 parasites/mL) and 4 (10 parasites/mL) from samples of culture, the participants were able to detect the presence of the parasite at both the high and low levels of parasite concentration. When the results were reported there were variations between the results that were reported for high-level *L. amazonensis* (836.78 parasites/mL) and *L. braziliensis* (638.39 parasites/mL) and for the low levels *L. amazonensis* (4,36 parasites/mL) and *L. braziliensis* (4.51 parasites/mL). That should to indicate that there were problems with the DNA recovery capacity of the samples. In the other hand, we had samples from DNA that showed a parasite load of 114.83 parasites/mL for *L. amazonensis* and

127.64 parasites/mL for *L. amazonensis*, indicating that the variation founded in the samples from culture is due to the variation of the efficiency of *Leishmania* recovery of the samples. The results of the qPCR were expressed in parasite equivalent in 1 mL show in table 7.

Table 7 Reproducibility of quantified of parasite load product from interlaboratory tests.

Participant	Sample 1	Sample 2	Sample 3	Sample 4
		From culture		
1*	0	1251.88	0	3.77
2⁺	0	1308.56	0	10.63
3⁺	0	171.16	0	2.28
4⁺	0	435.45	0	1.35
5*	0	421.68	0	4.94
		From DNA		
	Sample 1	Sample 2	Sample 3	
1*	0	0	170.59	
2⁺	0	0	106.39	
3⁺	0	0	134.96	
4⁺	0	0	103.14	
5*	0	0	84.68	

Samples from *L. amazonensis*; ⁺ Samples from *L. braziliensis

The data were analysed on base of detected and undetected a concordance between the results obtained and the nominal values of the samples with a p-value <0.05 ($p=8.45X10^{-18}$), in terms of relative sensitivity it was found with the test of proportion of true positives that the method developed is sensitive with a p-value >0.05 (p=1). Relative specificity with the true negative ratio test is determined that the method is specific with a p-value >0.05 (p=1). Finally, the relative accuracy was determined with the concordance test that the method is accurate with a p-value >0.05 (p=1).

5.4 VALIDATION OF qPCR IN VIVO

The assay was able to accurately measure a parasite load in the skin over different infection agents for cutaneous leishmaniases. Reactions with DNA from uninfected paws, included as negative controls did not amplify, and which indicates that wasn´t found false positive result. *Leishmania* resulted in an acute parasitemic phase easily detectable by qPCR under different infection of *Leishmania* species, in special the infection with *L. amazonensis* after 140 days post infection. In addition, 100% of samples from an animal infected with *Leishmania* were quantified despite the differences in symptoms caused by the different *Leishmania* species and no false negative (Fig. 6).

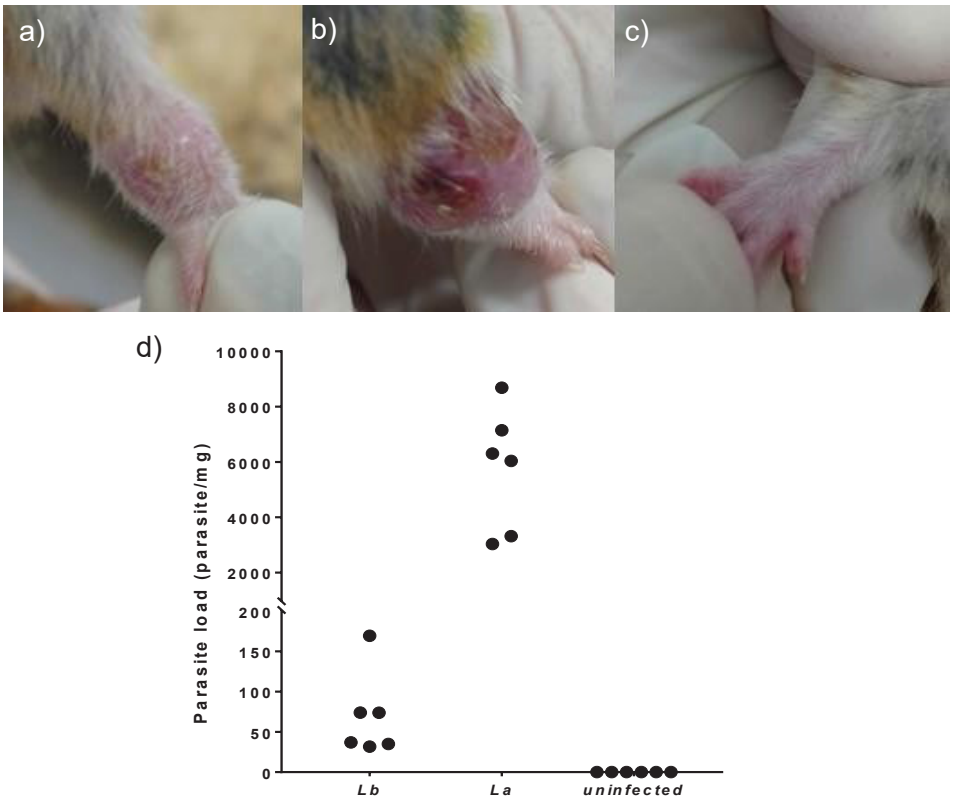

Fig. 6 Determination of parasite load in skin samples from an animal with or without experimental infection. The photograph shows the hamster's hind leg infected with different species of *Leishmania*. a) Animals infected with *L. braziliensis* (Lb). b) Animals infected with *L. amazonensis* (La). c) Animal uninfected. d) Parasite load of the samples.

5.5 EVALUATION OF PARASITE LOAD

In the microbiology and macroscopy evaluation of the endurance of the footpad lesion, it was possible to observe that 75 days post infection with *L. braziliensis* only one animal of the infected group had lesions characteristic of leishmaniases. At the same time it was also detected by microbiological culture, in the other groups, it was not detected the presence of *Leishmania*. At 120 days post infection, all animals in the infected group had characteristic lesions. Also, it was detected by the microbiological test. In the immunized group, only one animal had skin lesions. The uninfected group as expected was not injured in any study period (Table 8).

In the evaluation of parasite load in the spleen by qPCR, it was possible to observe 75 post infection with *L. braziliensis*. In all the groups no presence of *Leishmania* was detected. At 120 days post infection, all animals of the infected group were found to have *Leishmania* in the spleen, with a high variation of the parasitic load with values between $6.34X10^3$ to $9.19X10^6$ parasites/mg. A single animal from the immunized group was found to have *Leishmania* in the spleen with a parasite load of 6.95 parasites/mg. The uninfected group as expected was not detected with *Leishmania* in any study period (Fig. 7).

Table 8 Experimental groups and results relating to the presence of L. braziliensis in animal tissue after culture and quantification by qPCR.

Experimental groups	Clinical manifestation		N+/NT (% of infection)
	75 days post infection	120 days post infection	
Immunized	0/4 (0%)	2/5 (40%) detection in spleen and skin	2/9 (22.2)
Infected	1/4 (25%) detection in skin	4/4 (100%) Detection in spleen and skin	5/8 (62.5)
Uninfected	0/4 (0%)	0/4 (0%)	0/8 (0)

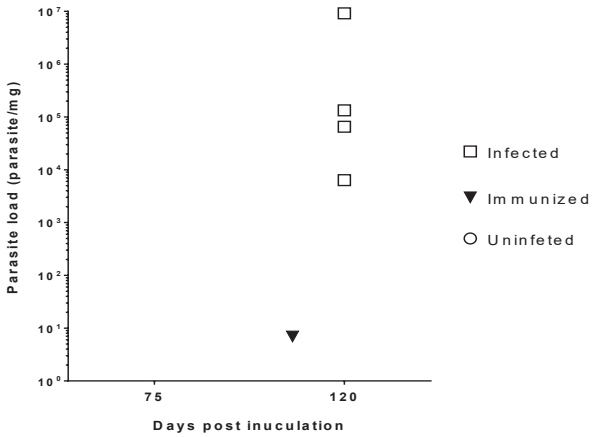

Fig. 7 Parasite load in the spleen of the group under study.

5.6 EVALUATION OF MRNA EXPRESSION PROFILE OF INOS AND CYTOKINES FROM SPLEEN

The cytokine expression profiles in *L. braziliensis* infected hamsters were analyzed by RT-qPCR. Expression profile was performed individually in each hamster, compared within groups using ANOVA with Tukey's HSD and interpreted as the fold change of mRNA levels in relation to the HPRT house-keeping gene (data not shown).

In the immunized group we found a possible induced effect 23.12 and 3.44 fold increased IFN-γ levels of mRNA in relation to the group infected at 75 and 120 days post infection respectively, 5.41 fold changes increased IL-12 levels of mRNA in relation of the infected group at 120 days post infection, 2.37 fold changes increased IL-4 levels of mRNA in relation of the group infected at 75 days post infection, then decreased to 0.65 times to 120 days post infection. Also found low levels of expression of IL 10 and TGF-β and TNF-α. In relation of iNOS had an increase in expression levels of 1.93 fold changes of mRNA more in relation to the infected group at 75 days post infection, then decreased to 0.64 times to 120 days post infection (Fig. 8).

In the infected group presented low levels of expression of interleukins, however, there was a high level of expression of IL-12 at 75 days post infection, which could have

stimulated increased expression IL-4, IL-10, and iNOS. Finally, for the uninfected group, there were no significant changes.

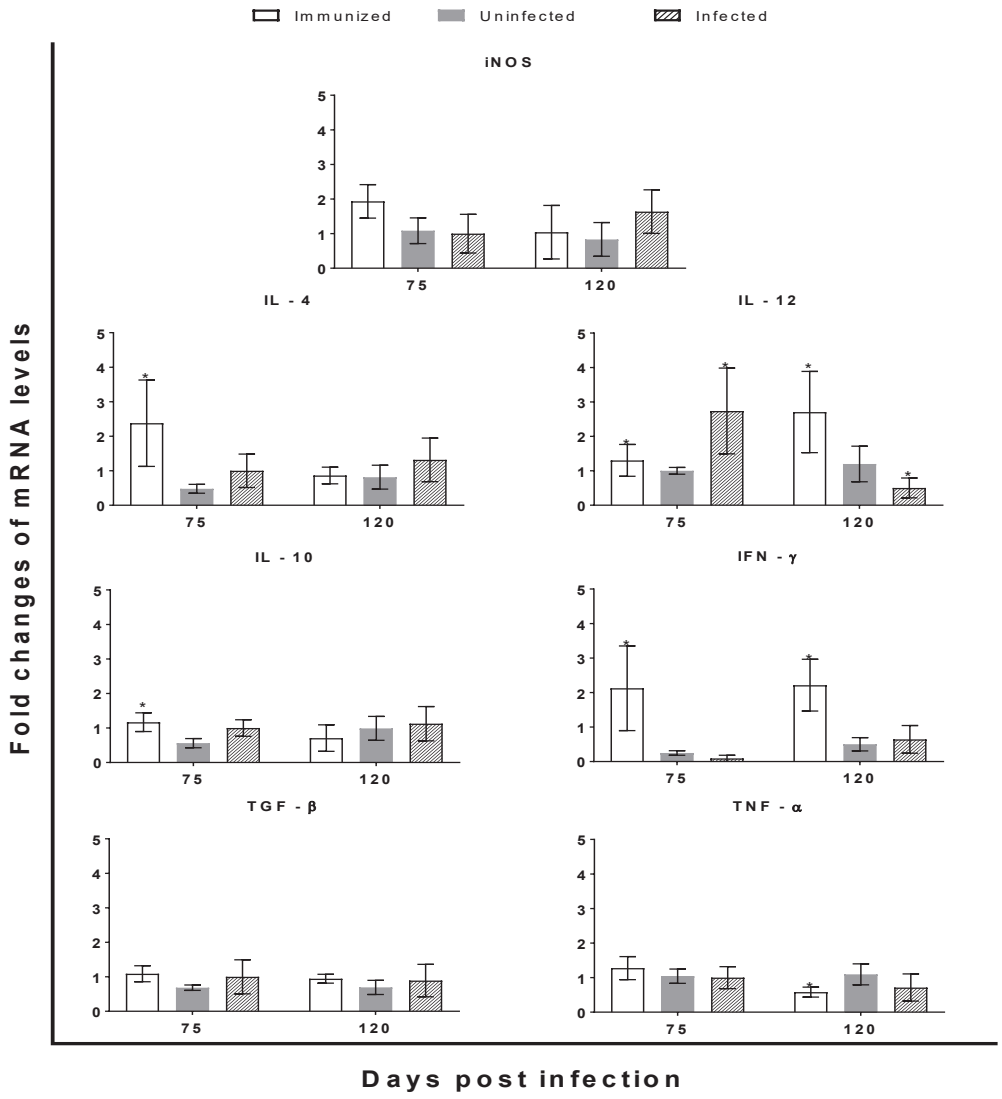

Fig. 8 Evolution of hamster's immune response to *L. braziliensis* infection with or without immunization. Data showed mRNA expression profile of iNOS, Th1 and Th2 cytokines (relative fold change) in *Leishmania*-infected animals treated with the product. Significance value (*p > 0.05) of treated cells was calculated in respect to untreated ones using ANOVA-Two ways. Bars represent as mean ± SD.

5.7 PRODUCTION OF IgG TOTAL AND IgG2a

The serum levels of leishmanial Ag-specific IgG total and isotypes IgG2a from all of the groups were assessed by ELISA. The anti-*Leishmania* IgG total and IgG2a were elevated progressively with time to a high level in all groups, except in the group product for IgG2a, in this case, they decrease the production for the 120 days post infection. In the group, unimmunized remained essentially the background levels (below of cut-off). In contrast, the group product showed a significant elevation by 2.9-fold for 75 days post infection to 3–fold to 120 over the uninfected group (p= <0.0001) in the level of IgG2a. The elevation of IgG2 was

consistent with the development of effective immune responses (Fig 9).

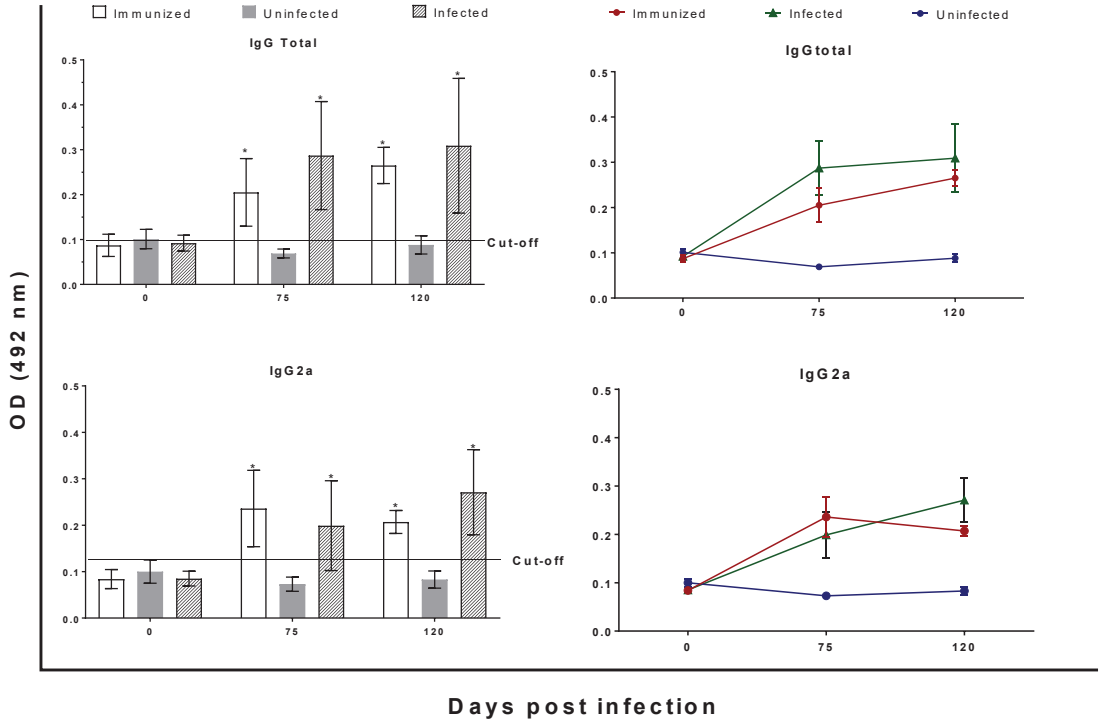

Fig. 9 Evolution of antibodies produced in hamster infected with *L. braziliensis* with or without immunization. Data showed antibodies expression IgG total and IgG2a. Significance value (*p > 0.05) was calculated in respect to the uninfected group. Bars represent as mean ± SD.

6. DISCUSSION

In research laboratories, PCR has been proposed to be an alternative tool for *Leishmania* sp. for quantification; it is more sensitive than the traditional parasitological techniques (ANTINORI et al., 2009; BENSOUSSAN et al., 2006; CECCARELLI et al., 2014; TORPIANO; PACE, 2015). However, it had disadvantages such as being time-consuming, the high risk of false positive results due to carry-over contamination or unspecific PCR products, and the difficult to perform quantitative analysis (PIRON et al., 2007).

In this context, the qPCR is emerging as an alternative tool for monitoring parasite load in experimental *Leishmania* sp. infections. However, there is no standardized qPCR protocol that is optimized specifically for use in animal models. In this work, we used two markers for parasite quantification. The kDNA that is traditionally the most used target for detection and identify *Leishmania*, because of its multicopy nature (the number of copies differs between species) and through high sensitivity (JARA et al., 2013). However, kDNA for the application parasite load is unpredictable because had a different relative abundance between species and is particularly unstable in terms of copy number in lifecycle stages of the parasite (CECCARELLI et al., 2017; MARY et al., 2004; SIMPSON et al., 2015;

WEIRATHER et al., 2011). The other hand, we had a marker of unique copy per genome which is less sensitive in relation to kDNA. But, is comparable between different species of *Leishmania*, because for a measure of parasite load it is required a process of correlation between marker copy per genome with the number of parasites. In addition, the use of single copy gene protocol while not being subject to changes copy number across lifecycle stages.

For a qPCR reaction is required the preparation of standards, usually used a genomic DNA from the target which includes a DNA extraction process, for determinate the number of copies, used the whole genome size of reference strains. However, this estimation may cause inaccurate quantification since the whole genome size might vary between strains. With the development of pDNApolyA allows us to have a stable size, speed up the process of quantification of the parasitic load and have a higher precision and be able to make the reactions of qPCR comparable among essays. We produced standardized negative controls and the development of novel pDNApolyA to be used in the proposed qPCR methodology, to later standardize and validate the qPCR technique in an animal model. In the same way, the validated technique was used to measure the parasitic load in vaccinated animals that were later experimental infected with *Leishmania*. To verify whether the quantification of *Leishmania* sp*.* the DNA would reflect the sensibility and number of live parasites present in the sample. The evaluation of the method allowed us to determine the sensitivity of the method which is 0.01 parasites per µL. In addition, the method showed to be reproducible by allowing classifying specimens as positive or negative was fairly uniform. The great advantage of qPCR is an application in monitoring of parasites in tissue samples (skin and spleen) with high sensitivity. Despite the advances in technology in the last few years, qPCR is far from being able to be used as a routine technique for the diagnosis or quantification of parasites, either because of the costs of reagents or equipment.

Since qPCR proved to be a versatile tool, was found to be a highly sensitive and reproducible technique for quantifying parasite load, also, it was used to quantify the immune response (Th1 and Th2) of vaccinated animals and compare them with the parasite load. As already known in the literature, *Leishmania* parasites are controlled by the Th1 cell response producing IFN-γ which activates or induces anti*Leishmania* effector pathways such as iNOS. It has been regulated by the expression of IL-12/IL-10 (BELKHELFA-SLIMANI and DJERDJOURI, 2018). The expression of IL-12 and IL-10 had an important role in the control or proliferation of *Leishmania* spp*.* IL-12 is pluripotent that play a central role in the initiation and maintenance of Th1 responses and IFN-γ production. On the other hand, IL-10 is a cytokine produced by Th2 that had a role to inhibition the expression of IL-12 and IFN-γ.

In the present study, the dosage of cytokine after 75 days post animals infections, a notable difference was already observed on the expression profile. The expression of IFN-γ

and IL-4 in the group who was immunized with the mix of peptides was significantly higher compared with control groups. Similar results were observed previously where the production of IFN-γ and IL-4 was detected in the spleen of mice (BIEDERMANN et al., 2001; HOCHREIN et al., 2000). This event would be linked to the fact that in the early stages of *Leishmania* infection IL-4 signaling on DCs during DC activation the production of IL-12 (HURDAYAL; BROMBACHER, 2014). In turn, would be reinforced with the concentration of IgG2a which is related to a Th1 cell response. Another study where mice were immunized with soluble leishmanial antigens (SLAs) from *L. tropica*, the authors founded high levels of IgG2a isotype of anti-*Leishmania* antibodies in this inbred mice strain associated with protective immunity against different *Leishmania* species (ROSTAMIAN et al., 2017). The IL-10 has an antagonistic function to the immune response type Th1 and is stimulated by the parasite to evade the immune system by inhibiting macrophage activation (BOGDAN, 2008). We found low levels of IL-10 expression in splenic tissue, over the first 75 days post infection. In the infected group, there was a slight increase in expression of IL-10 at 120 days post-infection which led to a decrease the expression of IL-12, this would be related to the detection of high levels of parasite load in the spleen in this group.

The absence of parasite in spleen at 75 days post immunization and the low concentration of parasites observed at 120 days post-infection in the immunized group, could be a result of the efficiency of the vaccine (peptide mix) having 77.8% protection against *L. braziliensis* is reflected in the increased expression of IL-12, IFN-γ and the decreased of IL-10 in spleen: The expression levels of these interleukins are an indication of a potent Th1-type polarized immune response elicited by the immunostimulatory ability of peptide mix, which would indicate peptide mix was able to provide considerable protection for hamsters against *L. braziliensis* challenge.

7. CONCLUSIONS

The methodology here developed, for quantification parasite load, by qPCR showed sensitivity and reproducibility;

The parasite load showed that the antigen used protected 77.8 % of the animal population from developing the infection, conferred by Th1 type of immune response exclusively.

8. PERSPECTIVES

- Test the methodology of qPCR with more participants and other laboratories.
- Development a control for determining the DNA extraction efficiency.
- Test the product in a larger group of individuals with different adjuvants and with shorter intervals of time between samples.

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SUPPLEMENTARY MATERIAL

ANNEX 1: Sequence used for the design of primer and probe DNA polymerase

Sequence accession no.	*Identification of sequence*
AF009134.1	*SauroLeishmania adleri* DNA polymerase alpha gene, partial cds
AF009135.1	*Leishmania aethiopica* DNA polymerase alpha gene, partial cds
AF009136.1	*Leishmania amazonensis* DNA polymerase alpha gene, partial cds
AF009138.1	*Leishmania braziliensis* DNA polymerase alpha gene, partial cds
AF009139.1	*Leishmania chagasi* DNA polymerase alpha gene, partial cds
AF009141.1	*Leishmania donovani* DNA polymerase alpha gene, partial cds
AF009143.1	*SauroLeishmania gymnodactyli* DNA polymerase alpha gene, partial cds
AF009146.1	*Leishmania hoogstraali* DNA polymerase alpha gene, partial cds
AF009147.1	*Leishmania infantum* DNA polymerase alpha gene, partial cds
AF009148.1	*Leishmania major* DNA polymerase alpha gene, partial cds
AF009149.1	*Leishmania mexicana* DNA polymerase alpha gene, partial cds
AF009150.1	*Leishmania panamensis* DNA polymerase alpha gene, partial cds
AF009151.1	*Leishmania tarentolae* DNA polymerase alpha gene, partial cds
AF009152.1	*Leishmania tropica* DNA polymerase alpha gene, partial cds
AF151728.1	*Leishmania enriettii* DNA polymerase gene, partial cds
AJ304942.1	*Leishmania turanica* partial dnap gene for DNA polymerase
AJ304943.1	*Leishmania major* partial dnap gene for DNA polymerase
AJ304944.1	*Leishmania guyanensis* partial dnap gene for DNA polymerase
AJ304945.1	*Leishmania gerbilli* partial dnap gene for DNA polymerase
CP009385.1	*Leishmania panamensis* strain MHOM/PA/94/PSC-1 chromosome 16 sequence
CP018582.1	*Leishmania donovani* strain MHOM/IN/1983/AG83 isolate early passage chromosome 16 sequence
CP019523.1	*Leishmania donovani* strain MHOM/IN/1983/AG83 isolate Late passage chromosome 16 sequence
CP022631.1	*Leishmania donovani* strain pasteur chromosome 16, complete sequence
CP027814.1	*Leishmania infantum* strain TR01 isolate Lin_TR01 chromosome 16, complete sequence
FR796412.1	*Leishmania major* strain Friedlin complete genome, chromosome 16
FR796448.1	*Leishmania infantum* JPCM5 genome chromosome 16
FR798990.1	*Leishmania braziliensis* MHOM/BR/75/M2904 complete genome, chromosome 16
FR799569.1	*Leishmania mexicana* MHOM/GT/2001/U1103 complete genome, chromosome 16
FR799603.2	*Leishmania donovani* BPK282A1 complete genome, chromosome 16
KJ667104.1	*Leishmania* sp. MHOM/CN/85/GS4 DNA polymerase alpha catalytic subunit (polA) gene, partial cds
KJ667106.1	*Leishmania* sp. MHOM/CN/89/GS5 DNA polymerase alpha catalytic subunit (polA) gene, partial cds
KJ667107.1	*Leishmania* sp. MHOM/GS/90/SC10H2 DNA polymerase alpha catalytic subunit (polA) gene, partial cds
KJ667109.1	*Leishmania* sp. MHOM/CN/83/GS2 DNA polymerase alpha catalytic subunit (polA) gene, partial cds
LN609207.1	*Leishmania peruviana* genome assembly *Leishmania* peruviana LEM-1537_V1, chromosome : 16
LN609244.1	*Leishmania peruviana* genome assembly *Leishmania* peruviana PAB-4377_V1, chromosome : 16
U78172.1	*Leishmania donovani* DNA polymerase alpha catalytic subunit gene, complete cds
XM_001464606.1	*Leishmania infantum* JPCM5 putative DNA polymerase I alpha catalytic subunit partial mRNA
XM_001563712.2	*Leishmania braziliensis* MHOM/BR/75/M2904 putative DNA polymerase I alpha catalytic subunit partial mRNA
XM_001682185.1	*Leishmania majo*r strain Friedlin putative DNA polymerase I alpha catalytic subunit partial mRNA
XM_003859800.1	*Leishmania donovani* DNA polymerase I alpha catalytic subunit, putative (LDBPK_161640), partial mRNA
XM_003873795.1	*Leishmania mexicana* MHOM/GT/2001/U1103 DNA polymerase I alpha catalytic subunit, putative partial mRNA
XM_010699377.1	*Leishmania panamensis* DNA polymerase I alpha catalytic subunit, putative partial mRNA

ANNEX 2: Sequence used for the design of primer and probe for kinetoplast

Sequence accession no.	*Identification of sequence*
AF103736.1	*Leishmania donovani* strain MHOM/SD/85/A22 kinetoplast minicircle DNA, complete sequence
AF103737.1	*Leishmania donovani* strain MHOM/SD/97/RLD1 kinetoplast minicircle DNA, complete sequence
AF103738.1	*Leishmania chagasi* kinetoplast minicircle DNA, complete sequence
AF103739.1	*Leishmania chagasi* strain MHOM/BR/74/PP75 kinetoplast minicircle DNA, complete sequence
AF103740.1	*Leishmania infantum* strain MHOM/UK/88/CILLONICZ kinetoplast minicircle DNA, complete sequence
AF103741.1	*Leishmania infantum* strain MCAN/TN/78/LEM78 kinetoplast minicircle DNA, complete sequence
AF167713.1	*Leishmania donovani* isolate MHOM/IN/82/NANDI-1 kinetoplast minicircle, sequence
AF168356.1	*Leishmania donovani* isolate MHOM/IQ/88/RTC6 kinetoplast minicircle, sequence
AF168357.1	*Leishmania donovani* isolate MHOM/SD/00/Khartoum kinetoplast minicircle, sequence
AF168358.1	*Leishmania donovani* isolate MHOM/CN/80/STRAIN-A kinetoplast minicircle, sequence
AF169131.1	*Leishmania infantum* strain MCAN/PT/88/REBELO2 kinetoplast minicircle DNA, complete sequence
AF169134.1	*Leishmania donovani* strain MHOM/SD/95/MSA2 kinetoplast minicircle DNA, complete sequence
AF169135.1	*Leishmania donovani* strain MHOM/SD/97/RHD-48 kinetoplast minicircle DNA, complete sequence
AF169136.1	*Leishmania donovani* strain MHOM/SD/95/SIGIN kinetoplast minicircle DNA, complete sequence
AF169137.1	*Leishmania chagasi* strain MHOM/PA/79/WR317 kinetoplast minicircle DNA, complete sequence
AF184044.1	*Leishmania infantum* isolate MCAN/IT/80/ZORRO kinetoplast minicircle DNA, complete sequence
AF190476.1	*Leishmania infantum* isolate MHOM/SU/84/MARZ-KRIM kinetoplast minicircle DNA, complete sequence
AJ010074.2	*Leishmania donovani* kinetoplast minicircle DNA, isolate MHOM/IQ/88/RTC6, clone 11
AJ010075.2	*Leishmania donovani* kinetoplast minicircle DNA, isolate MHOM/IQ/88/RTC6, clone 17
AJ010077.2	*Leishmania donovani* kinetoplast minicircle DNA, isolate MHOM/SD/85/FORSTER, clone 14
AJ223724.1	*Leishmania infantum*. Minicircle DNA of L. infantum isolated from a kala-azar patient (778 bp)
AJ270145.1	*Leishmania donovani* kinetoplast minicircle DNA, strain LEM 703
AJ275327.1	*Leishmania infantum* kinetoplast partial minicircle DNA, strain MHOM/ES/97/LLM-719, clone 572
AJ275332.1	*Leishmania infantum* kinetoplast partial minicircle DNA, strain MHOM/ES/97/LLM-719, clone 577
EU370887.1	*Leishmania infantum* isolate Li-SP-52 kinetoplast minicircle, partial sequence
EU370905.1	*Leishmania major* isolate Lm-FR-20 kinetoplast minicircle, partial sequence
FJ416603.1	*Leishmania donovani* isolate MHOM/SD/62/1S-Cl2D maxicircle, partial sequence; kinetoplast
FR799614.1	*Leishmania donovani* BPK282A1 complete genome, chromosome 27
HF563611.1	*Leishmania infantum* mitochondrial non-protein coding region, isolate 4a
HF563612.1	*Leishmania infantum* mitochondrial non-protein coding region, isolate 5a
KM555288.1	*Leishmania major* strain MHOM/IL/67/LV561 minicircle, complete sequence; kinetoplast
KM555295.1	*Leishmania major* strain MHOM/IL/67/LV563 minicircle, complete sequence; kinetoplast
KU220265.1	*Leishmania donovani* isolate Ld_NP-PKDL_BPK-PKN466-8 minicircle kinetoplast, complete sequence
L19877.1	*Leishmania donovani* minicircle sequence
U51720.1	*Leishmania major* kinetoplast DNA sequence III
Y11401.1	*L.donovani* kinetoplast minicircle DNA, 792 bp
Z35269.1	*L.infantum* (AJS-IPTBG) kinetoplast DNA
Z35274.1	*L.infantum* (AJS-IPTRS) kinetoplast DNA
Z35276.1	*L.chagasi* (AJS-PPECO) kinetoplast DNA
Z35292.1	*L.infantum* (AJS-D2PST) kinetoplast DNA
Z35500.1	*L.infantum* (MCAN/ES/97/LLM-32) kinetoplast DNA, 767 bp
Z35501.1	*L.infantum* (MCAN/ES/87/LLM-32) kinetoplast DNA, 774 bp