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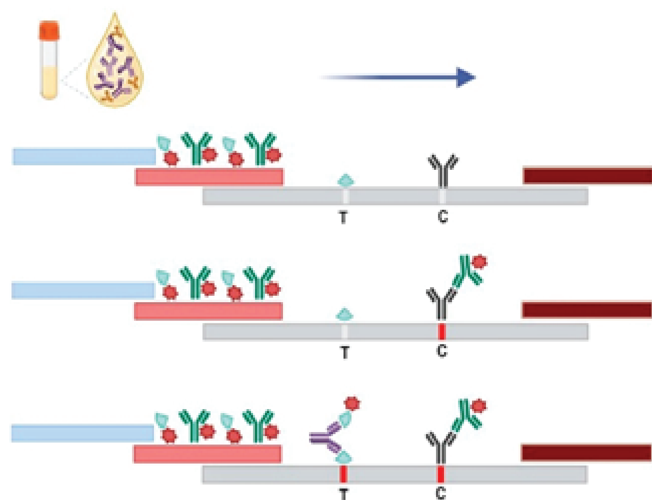
Identification of NEW ANTIGENS for the diagnosis of VISCERAL LEISHMANIASIS

APPLICATION IN IMMUNOCHROMATOGRAPHY



WILLIANE FERNANDA SIQUEIRA

Maastricht University

Identification of New Antigens for the Diagnosis of Visceral Leishmaniasis - Application in Immunochromatography

DISSERTATION

to obtain the degree of Doctor at Maastricht University, on the authority of the Rector Magnificus, Prof. Dr. Pamela Habibović in accordance with the decision of the Board of Deans, to be defended in public on Tuesday the 19th of September 2023, at 10:00 hours

by

Williane Fernanda Siqueira

Supervisors:

Prof. Dr. Thomas Jan Cleij

Prof. Dr. Ricardo Toshio Fujiwara

Co- supervisor:

Dr. Bart van Grinsven

Assessment committee:

Prof. Dr. Leon Claessens (Chair)

Prof. Dr. Patricia Maria Losada Perez

Prof. Dr. Laia Solano-Gallego

Prof. Dr. Ricardo Nascimento Araujo

Prof. Dr. Mauricio Sant'Anna

Dr. Carmen Padilla Diaz

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Identification of New Antigens for the Diagnosis of Visceral Leishmaniasis - Application in Immunochromatography

I dedicate this work to my grandmother Isabel Josafá,
to my mother Mara da Conceição Souza,
to my father Roberto Francisco Siqueira
and to my uncle Daniel Cesar de Souza.

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List of abbreviations

AC – Accuracy
Asy – Asymptomatic
AUC – Area Under the Curve
Bab – *Babesia* sp.
BCA – Bicinchoninic Acid Solution
BLAST – Basic Local Alignment Search Tool
BSA – Bovine Serum Albumin
CAAE – Certificate of Presentation of Ethical Appreciation
CanL – Canine Leishmaniasis
CEUA – Ethics Committee on the Use of Animals
CI – Confidence interval
Co-Infec – Co-Infected
CSA – Crude Soluble Antigen
DAT – Direct Agglutination Test
DCM – Dichloromethane
DIC – Diisopropylcarbodiimide
DMF – Dimethylformamide
DNA – Deoxyribonucleic Acid
DPP – Dual Path Platform
ELISA – Enzyme-Linked Immunosorbent Assay
Erh – *Ehrlichia* sp.
FN – False Negative
HVL – Human Visceral Leishmaniasis
ICT – Immunochromatographic Test
IFA – Indirect Immunofluorescence Reaction
Ig – Immunoglobulin
IgG – Immunoglobulin G
IPTG – Isopropil-β-D-1-Tiogalactopiranosido
kb – Kilobyte
kDa – Kilodalton
KDDR – Kinesin Degenerated Derived Repeat
kDNA – Kinetoplast DNA
LR – Llikelihood
MIP – Molecularly Imprinted Polymers
NC - Negative Control
NCBI – National Center for Biotechnology Information
NI – Not Infected
NPV – Negative Predictive Value
ORF – Open Reading Frame
pb – Base Pairs
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
pI – Isoelectric Point
PPV – Positive Predictive Value
qPCR – Quantitative Polymerase Chain Reaction

ROC – Receiver Operating Characteristic
SDS-PAGE – Polyacrylamide Gel Electrophoresis of Sodium Dodecyl Sulfate
Sen – Sensitivity
Spe – Specificity
Sym – Symptomatic
TFA – Trifluoroacetic Acid
TIPS - Triisopropylsilane
TP – True Positive
TR – Tandem Repeat
VL – Visceral Leishmaniasis
WHO – World Health Organization

1. Chapter I - Initial considerations and contextualization

Visceral leishmaniasis: historical contributions and advances in diagnosis for disease control.

Adapted from:

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1.1. Chapter context

This initial chapter will provide an overview of visceral leishmaniasis (VL) and its impact on public health in Brazil. In addition to bringing conceptual clarifications about the disease, this chapter also addresses the main historical contributions, since the almost accidental discovery of the disease in Brazil by Penna, in the early 1930s, until the present day. The trajectory of the diagnosis, together with the advances obtained over almost 9 decades, were chronologically traced and explored in order to present the advantages and disadvantages or limitations of the main techniques used in the diagnosis of VL. The rapid progress, development, and improvement of diagnostic techniques for VL in a relatively short period, especially in the last decade, highlights the important role of diagnosis in disease control.

1.2. Chapter abstract

Visceral leishmaniasis (VL) is an important infectious disease that integrates a complex group of parasitic diseases, causing great socioeconomic impact, morbidity and mortality in Brazil and worldwide. VL is considered the most severe form among the different types of leishmaniasis, and it can lead to the death of individuals affected in 90% of cases, if not diagnosed and treated. In Brazil, VL is a vector-borne and zoonotic disease, with domestic dogs as primary reservoirs in the urban environment. Among the disease control measures, the Ministry of Health of Brazil recommends the elimination of infected dogs, since the human infection is preceded by the canine infection, in addition to the early diagnosis and the treatment of human cases. Therefore, a safe and accurate serological diagnosis is necessary, since an erroneously positive result can lead to the wrong treatment of uninfected patients and a false negative result allows the progression of the disease and, consequently, the patient's evolution to death. The discovery and use of recombinant targets have improved the performance of the main techniques used in the diagnosis of the disease. However, there is a need to associate the search for new molecular targets with the development of new diagnostic platforms, such as biosensors, which also allow the use of different biological samples, such as blood, serum, plasma and urine. Taken together, these strategies will be able to overcome the existing barriers of currently available diagnostic methods, being able to contribute to the control of VL through a more efficient and reliable test.

1.3. Introduction

Visceral leishmaniasis (VL) is a systemic infectious disease with a chronic course, caused by intracellular parasites belonging to the *Leishmania donovani* complex, which includes the species responsible for the visceralization of the disease, such as *L. (L.) donovani* and *L. (L.) infantum* (1). The transmission of VL occurs through the bite of female hematophagous insects, belonging mainly to the genera *Phlebotomus* and *Lutzomyia*. In Brazil, as in other New World countries, the disease

has a zoonotic character, with domestic dogs (*Canis familiaris*) being considered the main source of *Leishmania* infection, playing an important role in maintaining the cycle in the urban environment and in transmitting the disease (2).

The visceral form of the disease, also known as kala-azar, is considered the most severe form of leishmaniasis, being characterized by long periods of fever, weight loss, anemia and changes in internal organs such as the spleen and liver, which leads to death of affected individuals in 90% of cases, if not diagnosed and treated (3). In addition, VL is on the list of neglected tropical diseases and persists as a serious public health problem, which affects millions of people worldwide (4).

In Brazil, the disease is endemic and its clear territorial expansion to municipalities has led to a number of undiagnosed cases (3). The urbanization of *Lutzomyia longipalpis*, the main vector of *L. infantum* in the Americas, played a central role in the disease spread in urban areas. This process of vector urbanization was first detected in Teresina the capital of Piaui State, Brazil, spreading to other urban centers (5). In urban settings, the vector *L. longipalpis* highly susceptible and permissive and infects dogs, especially stray dogs, that are efficient *L. infantum* reservoirs and play a central role in the spread of the disease. According to data from the Ministry of Health (2019) from 2010 to 2018, around 30,666 cases of human VL (HVL) were confirmed, of which 2,292 progressed to death (6). Although the data on the disease are alarming in Brazil, surveillance and control measures for this zoonosis are in place, with emphasis on early diagnosis, followed by adequate treatment of human cases.

From this perspective, the development of accurate and precise diagnostic tests, which present sufficient sensitivity to correctly identify human cases and infected dogs, minimizing the number of false negative cases, and which have satisfactory specificity, allowing the correct discrimination of individuals and avoiding false positive results, is extremely desirable. This would facilitate the diagnosis of HVL and, consequently, access to treatment would be faster and more accurate. It is worth remembering that the diagnosis for canine leishmaniasis (CanL) can help in the control of infection in dogs and, therefore, in the reduction of human infection, since canine enzootic disease precedes the appearance of human cases and the infection of dogs has been more prevalent than infections in humans (7).

1.4. Historical contributions of visceral leishmaniasis diagnosis

In 1934, when Henrique Penna unexpectedly reported the discovery of the parasite *Leishmania* sp. in human post-mortem histological sections, followed by the first human *in vivo* diagnosis by Evandro Chagas in 1936, epidemiological studies of VL in different regions of the country began (8). Consequently, a few years later, the first surveillance and control actions for the disease started to be directed to the main agents involved in the transmission cycle of the disease, such as insect vectors, canine reservoirs and human cases, with emphasis on the early identification of individuals infected with *Leishmania*.

Since the 1930s, the diagnosis of VL has been based on a triad composed of clinical, epidemiological and laboratorial aspects. The diagnosis by laboratory tests is

usually associated with clinical and epidemiological parameters, since the classic symptoms of VL are similar to the clinical picture of other diseases (7).

1.4.1. Clinical diagnosis

In humans, VL presents several signs and symptoms observed in other pathologies, which makes the clinical diagnosis complex and subject to uncertainties. These symptoms may vary from patient to patient, depending on factors such as the type of strain present and immunological aspects of the patients. Thus, there are asymptomatic individuals and those who present fever and splenomegaly associated or not with hepatomegaly and pancytopenia (6). In turn, the disease in the canine population is asymptomatic in most cases, but when signs are present, they vary from mild skin changes, alopecia and apathy to severe signs characterized by lymphoid adenopathy, anorexia, increased size of the spleen and liver, onychogryphosis and keratoconjunctivitis (6).

1.4.2. Laboratory diagnosis

The laboratory diagnosis of leishmaniasis is made by interpreting the results of blood tests, such as hemograms or protein dosage, parasitological, serological or molecular tests, each with advantages and limitations inherent to the method (7). However, none of these tests has proven sufficiently safe, accurate, practical and fast, simultaneously compromising the efficiency of disease control (9).

Complementary Laboratory Diagnosis

A blood count in patients with VL is characterized by pancytopenia, that is, a decrease in the count of red blood cells, leukocytes and platelets, as in other infectious and parasitic diseases. Regarding the protein dosage, there is a strong inversion of the albumin and globulin ratio marked by hyperglobulinemia (7). However, these data alone are not sufficient to elucidate or not the presence of the disease in an individual.

Parasitological Examinations

Direct parasitological methods, based on direct microscopic visualization or parasite culture performed through biopsy material from aspiration puncture of the spleen, bone marrow or lymph nodes, have been considered the gold standard for the diagnosis of VL since the 1930s, remaining so today (1). These methods have specificity levels that can reach 100% (7). However, their sensitivity is very variable (60 to 95%), because the distribution of parasites is not homogeneous in tissues and the detection capacity is dependent on the parasite load of the individual and the material analyzed (10). In addition, the collection of biological material for parasitological diagnosis is made through invasive, painful and risky techniques for patients. Therefore, they have limited applicability in the field due to the risks

generated to individuals and the need for a qualified technical workforce both for the collection of material and for the interpretation of microscopy (1).

Molecular Methods

Diagnostic methods using molecular techniques to identify parasites of the genus *Leishmania* have been suggested since the 1980s (1). The use of these molecular techniques has become increasingly indispensable due to their good accuracy and the possibility of using peripheral blood, serum and urine samples, thus avoiding invasive methods such as aspiration of bone marrow, lymph nodes, spleen and liver (1). Polymerase Chain Reaction (PCR) techniques have been used in the diagnosis of leishmaniasis, with conventional PCR currently being the most widely employed diagnostic tool (11). The sensitivity, specificity, precision and reproducibility of molecular techniques can vary depending on the choice of biological tissue or target used, which is a potential drawback of using molecular diagnostics (1). However, a sensitivity of up to 94% can be easily obtained (7). PCR can also be used for patient healing follow-up during treatment or post-treatment. Despite the technological evolution of PCR, for the public health service, this technique is costly, requiring sophisticated equipment and qualified professionals. Thus, this type of diagnosis has less applicability in the field and restricted use in mass screening of the population (12).

Serological methods

An alternative to invasive parasitological methods are serological assays, since VL is characterized by a large production of specific antibodies along with polyclonal B-cell stimulation (7). A vast number of serological techniques are available for the diagnosis of the disease, enabling a wide spectrum of variations in sensitivity and specificity, cost-effectiveness, and field and mass applicability (1, 9). The serological techniques most commonly employed in the diagnosis of VL are: the indirect immunofluorescence reaction (IFA), the direct agglutination test (DAT), the enzyme-linked immunosorbent assay (ELISA), and, more recently, immunochromatographic devices, known as the rapid immunochromatographic test (ICT) (1).

The IFA was first used in the 1980s and since then has gradually developed into the gold standard for the confirmation of infection by *Leishmania* (13). This technique is based on the search for antibodies through the recognition of whole *Leishmania* promastigote antigens. The IFA has been widely used in population surveys and has a sensitivity that can range from 82 to 95% and a specificity from 78 to 92% (14). However, it is an assay of subjective interpretation, dependent on the experience of the microscopist, and it requires relatively sophisticated equipment, which makes it laborious, expensive and difficult to reproduce in the field (14). Although this technique is currently still used for the diagnosis of HVL, in 2012, the Ministry of Health changed the protocol used in canine epidemiological surveys, replacing IFA with the introduction of the ICT (15).

Developed by El Harith *et al*. (16), the DAT detects antibodies in biological fluids in the presence of fixed promastigotes. The method is based on the fact that after a certain period of incubation, an agglutination reaction visible to the naked eye is observed. DAT is a simple, easily performed and relatively inexpensive method. A systematic review by Chappuis *et al.* (17), showed that this method has a sensitivity of 94.8% and specificity of 85.9%. Although this is a simple test, the disadvantages of this method are the loss of precious time during the incubation period, the need for multiple pipetting, and the inability to differentiate between clinically active and asymptomatic infections that provide a positive result, even for a long time after cure (1).

Introduced in the 1970s by Hommel *et al*. (18), the ELISA is one of the most used serological tests today, both for human and canine diagnosis. This technique is advantageous because it is a relatively simple and fast tool that can be performed manually or in automated fashion, besides allowing the analysis of a large number of samples simultaneously in a short period of time (1). The ELISA allows the use of different types of antigens, such as crude or soluble extract of *Leishmania* or recombinant proteins. However, its sensitivity and specificity varies according to the antigen used (14). In addition, the use of crude antigen in this technique has proven to be a factor limiting its specificity. In this context, recombinant proteins have emerged as an alternative in ELISA-based serological diagnostics. Several recombinant *Leishmania* antigens have been characterized and tested in order to improve the serodiagnosis of leishmaniasis by ELISA. Among them, the recombinant antigen K39 (rK39), showed the most promising results for the diagnosis of the disease (19). Like the IFA, the ELISA is also dependent on skilled labor for interpretation of results and equipment that has not yet been optimized for field use. Another disadvantage of the technique is the possibility of cross-reactions with other diseases, such as Chagas disease, schistosomiasis, among others (7).

In turn, the ICT is a qualitative test that is mostly based on the detection of antibodies in serum, plasma or blood, through recombinant antigens fixed on a cellulose strip (2). Featuring a sensitivity of 67 to 100% and specificity of 70 to 100%, this test was developed to facilitate the diagnosis of the disease *in situ* (20). Currently, a number of commercial ICT kits are available, which have been properly standardized and validated for both field and laboratory use (21). The low-cost, easy execution, fast result, and the independence of technology on skilled labor have made this test an excellent tool for mass screening. However, cross-reactivity with other parasites and other diseases is reported, as well as difficulty in detecting asymptomatic individuals (2).

1.5. Recent advances in the diagnosis of visceral leishmaniasis

1.5.1. Main recombinant proteins for use in serological tests for VL

Recently, research on VL diagnosis has been directed towards further technological innovation of immunoassays. As mentioned earlier, a lot of progress in this field can be made by designing more sensitive and specific recombinant molecules to avoid false negative and false-positive results, both for screening and confirmatory diagnosis of the disease. Among the advantages of using recombinant

molecules, one can mention the large-scale production of a standardized form of antigen and independent of the growth of *Leishmania* in laboratory cultures (2).

Recombinant antigens emerge as an alternative to improve the serological methods currently used, such as DAT, IFA and ELISA, because they are molecules of the parasite that have a great affinity with antibodies present in most biological samples (2). Over the years, several recombinant proteins from *L. infantum* and *L. donovani*, with varying sensitivity and specificity, have been characterized and evaluated, proving to be useful in the discrimination of VL (Table 1).

Table 1 - Main antigens described in the literature for use in serological tests for VL.

Antigen	Species	Protein origin	Source	Sen. (%)	Spe. (%)	References
rK39	*L. infantum* (syn. *L. chagasi*)	Kinesin related protein	Brazil	26.5 – 100 (ELISA) 67.0 –100 (ICT)	73.7 – 100 (ELISA) 70.0 – 100.0 (ICT)	(19)
rA2	*L. donovani*	Amastigote specific protein	Canada	62.7 – 100	77.59 – 100	(22)
rK9	*L. infantum* (syn. *L. chagasi*)	Hydrophilic Protein	Brazil	78,0	84.0	(23)
rK26	*L. infantum* (syn. *L.*	Hydrophilic Protein	Brazil	38.0 – 100	90.0 – 100	
rKE16	*L. donovani*	Kinesin	Índia	32.4 – 100	92.9 – 100	(24)
KRP42	*L. donovani*	Kinesin	Japan	94.4 – 94.6	99.3 – 96.0	(25)
rK28	*L. donovani*	HASPB1/LdK39/ HASPB2	Sudan	90.6 – 100	56.4 – 100	(26)
KLO8	*L. donovani*	Kinesin related protein	Germany	96.2 – 100	96.1 – 100	(20)
rKR95	*L. donovani*	Kinesin related protein	USA	66.0	89.5	(27)
rKDDR	*L. infantum* (syn.	Kinesin related protein	Brazil	88.5 – 92.9	97.3 – 100.0	(28)

Syn.: synonym, Sen.: sensitivity, Spe.: specificity, ELISA: Enzyme-Linked Immunosorbent Assay, ICT: rapid immunochromatographic test.

The race for new antigenic molecules directed to the diagnosis of VL began in the early 1990s, when Burns *et al*. (19) characterized an antigen related to the kinesin protein superfamily of *L. infantum* for the improvement of human and canine diagnosis. Kinesins are part of a superfamily of motor proteins, which are involved in the process of mitosis and flagellum movement in *Leishmania* species and are also responsible for vesicle locomotion (29). They evaluated a 230 kDa antigen, called LiKin, related to the kinesin molecule, which is encoded by a gene from *L. infantum* (tag locus: Linj.14.1180) and is conserved among viscerotropic *Leishmania* species (19). From a fragment of this antigen, a recombinant protein, rK39, was generated and characterized, containing a highly immunogenic epitope that presents a series of repetitive portions of 39 amino acids. Badaró *et al.* (30) characterized and evaluated

rK39 against lysed *Leishmania* crude antigen for antibody detection and obtained results that confirmed the highly specific nature of rK39 with 99% sensitivity and 100% specificity. The promising relationship of rK39 with VL diagnosis is associated with the presence of an abundant repetitive region in its protein sequence, which apparently makes it highly antigenic (19, 20, 23, 26). However, validation studies of this antigen have shown variable performance results, according to the geographical region of research and methodology employed (1). Despite the good performance of rK39 obtained in countries like Nepal and India (31, 32), in countries like Brazil and others in East Africa, its performance is still low (33).
Another antigen tested is the hydrophilic K26 protein which presents 11 repeats of a 14 amino acid sequence (23). The rK26 has a sensitivity ranging from 38 to 100% and a specificity of 90 to 100% (11, 26, 32, 34).

Recently, the recombinant KDDR antigen was described by Dhom-Lemos *et al.*, which is predominantly based on the repetitive portion found in the kinesin protein of *L. infantum*. Whereas, the rK39 protein is composed of two parts, one being non-repetitive and the other repetitive, with approximately 6.5 motifs of 39 amino acids, rKDDR is composed of a recurrent sequence of 8.5 repetitive blocks of 39 amino acids from kinesin, which corresponds to 92% of the total sequence of the protein (28). This new protein showed promise when compared with the rK39 antigen in ELISA tests. Human serological recognition against rKDDR using ELISA showed higher sensitivity and specificity (92.4% and 100.0%, respectively) when compared to the rK39 ELISA test, which showed sensitivity and specificity of 90.5% and 97.9%, respectively. In turn, for the canine diagnosis through ELISA, the assay with rKDDR showed a higher sensitivity (88.5%) and specificity (97.3%) when compared to rK39, which showed sensitivity of 78.1% and specificity of 90.1%.

Thus, although there are numerous recombinant antigens available for the diagnosis of VL with good efficacy and despite the continued use of the promising rK39 antigen, none of them have proven 100% effective in the serodiagnosis of this disease, indicating the need for improvement of antigens capable of providing a result of greater reliability (9). Furthermore, the development of new platforms that improve and amplify the performance of recombinant proteins is necessary.

1.5.2. Diagnostic tests using urine as biological sample

Approximately two decades ago, the first report of the development of a test to detect leishmanial antigens in the urine of VL patients, a new latex agglutination test (KAtex), appeared (35). This study opened new perspectives for the development of tests for VL, because during the disease several proteins produced by *Leishmania* are metabolized and eliminated through host urine. Since then, several antigen detection test have been described for VL diagnosis using patient urine, including ELISA, DAT, KAtex and ICT (Table 2) (36). The main advantage of urine tests is the ease of obtaining the material. Samples can be collected several times without causing discomfort to the patient, they do not require processing, the involved in collecting the material is low, and the procedure is less invasive and risky to the patient (12).

Table 2 - Sensitivity and specificity of diagnostic tests for VL using urine as the biological sample.

Diagnostic test	Sen. (%)	Spe. (%)	References
KAtex	68.0 - 100	100	(35)
ELISA	93.5 - 95.0	89.3 - 95.3	(37)
DAT	90.7	96.4	(37)
PCR	97.0	100	(38)
rK39	95.0	93.3	(39)

Sen.: sensitivity, Spe.: specificity.

Unlike “conventional” serological tests, where the target molecules is the antibodies, when urine is used as a sample, the targets are normally the antigens secreted by the parasite. This inversion of targets, in principle provides better means for the diagnosis of VL because it overcomes the major problem associated with the detection of antibodies: the fact that individuals co-infected with Human immunodeficiency virus (HIV) or individuals with infection in the early or asymptomatic phase have low antibody response to the antigens of the parasite (35).

It should also be considered that a test in which the detection of antigens and not the identification of antibodies is performed may assist in the differentiation of active cases from previous exposures to the parasite or even help in monitoring the therapeutic efficacy of VL patients (12). Samples from patients who have had leishmaniasis and currently receive treatment or even those who have already finished treatment, may present "memory antibodies" during a certain period which, in turn, may be detected in "traditional" serological tests, resulting in false-positive reports or even indicating the patient's non-cure post-treatment (12).

However, the use of urine as a clinical sample for the diagnosis of VL is not only limited to serological tests, but is also used for the extraction of nucleic acid from the parasite for use in the molecular diagnosis of the disease and for the culture of *Leishmania* isolated from patient urine (40). Da Costa Lima *et al.* (41) showed that out of 30 samples from patients with visceral leishmaniasis, 6 had *Leishmania* DNA. Furthermore, in this same study, promastigotes were isolated in culture from the urine of one patient.

Although urine has become a valuable biological sample for the diagnosis of HVL, it is still not widely used because it has limitations such as the presence of debris and inhibitors of PCR amplification, lack of an efficient method of urinary DNA extraction, and the low number of parasites present in urine (40). Such limitations may explain, but not justify, the lack of a simple, rapid, and less invasive commercial kit that can be used both in laboratories as well as reference centers or places with limited resources.

1.5.3. Immunobiosensors

In addition to the relentless search for new molecules to be used in existing platforms (9), it is necessary to direct resources and research toward the discovery of different techniques that are as practical as a rapid test with higher accuracy than an ELISA and that enable the use of these molecules.

Recently, several studies have been directed to the development of biosensors, typically immunobiosensors that use antibodies to capture their target. Antigen-antibody interaction is translated into an interpretable signal by various transducer techniques based e.g. on electrochemical or piezoelectric detection, chromatographic techniques of molecules, among others (42-44). These techniques are also compatible with a wide range of other biological recognition elements, capable of detecting substances or chemical components. In short, these biosensors are, for the most part, based on the same principle as immunoassays: the interaction between antigen/antibody (enzyme-substrate, target-receptor…), do not only lead to a qualitative but also a quantitative result (42).

One study, conducted by Cordeiro *et al.* (44), in which an electrochemical impedance immunosensor, using screen-printed electrodes (SPEs) for the detection of anti-*L. infantum* antibodies, was shown to be sensitive, specific, rapid and simple, with potential application for the serological diagnosis of VL. In another research, a surface plasmon resonance (SPR) immunosensor was developed, using a gold sensor chip modified with 11-mercaptoundecanoic acid (11- MUA), also showed promising results in the identification of anti-*L. infantum* antibodies (43). Another quartz crystal microbalance immunosensor, using a recombinant *L. chagasi* antigen (rLci2B-NH6), has been studied for use in the diagnosis of CanL, being able to distinguish canine sera positive and negative for *L. chagasi*, besides showing good results 14 as to sensitivity and reproducibility (45). A bioelectronic device capable of rapidly detecting *Leishmania* sp. antigens was proposed by Cabral-Miranda *et al*. (42), being able to detect 1.8×10^4 amastigotes/g of infected tissue.

Following the trend of research directed to biosensors, the research group of the Department of Sensor Engineering at the Faculty of Science and Engineering at the Maastricht University has been seeking to develop an immunosensor capable of detecting analytes from a given sample by measuring the blockage of heat transport in a thermocouple circuit, properly recorded in an isolated compartment. This new biosensor is based on so-called MIPs (molecularly imprinted polymers), which are synthetic receptors that have recognition sites for the complementary molecules of interest in terms of size, shape, and orientation of functional chemical groups, allowing the identification of various molecules (46). In this way, these MIPs mimic the affinity that biological receptors have for their targets and can bind them in a manner similar to an antibody-antigen interaction. Biosensors using MIPs overcome many issues associated with the use of biological receptors due to their long-term chemical and thermal stability, as well as having a low-cost synthesis process (47). This new diagnostic tool emerges as an alternative to the current diagnostic platforms available, being economically feasible to be able to target the population deprived of resources and, consequently, the most affected by neglected diseases such as VL.

In view of the numerous examples mentioned above, it is evident that the scientific interest in biosensors is continuously growing and the successful results summarized in literature indicate that biosensors hold great promise as sensitive, specific, fast and simple detection systems, with potential application in the field or laboratory for the serological diagnosis of VL.

1.6. Final considerations

Currently, despite the availability of ICT for VL diagnosis, standard procedures are still based on antigens selected through conventional methodologies. The resulting diagnostic tests still display a limited performance (sensitivity and specificity), which could be improved by introducing new methodologies into diagnostic procedures. Therefore, investment policies in research and technological development of new diagnostic platforms, as well as the search for and improvement of new targets for the diagnosis of VL are both essential approaches to control this disease more quickly and effectively. The correct diagnosis can allow the immediate initiation of treatment and epidemiological surveillance on a larger scale, assisting in clinical and epidemiological decisions and thus promoting better control actions of the disease.

However, for biosensors to actually reach the market, the interest and contribution of the public and private sectors is necessary for the registration and validation of new diagnostic tests. Therefore, there is a growing need for multicenter studies to evaluate the efficacy of new tests. These steps are still largely missing in this field but are crucial for new tests or new diagnostic platforms to grow past the stage of academic literature and to actually reach the stage of commercial development and be introduced in the market. Only then can these new technologies truly accelerate the field of VL diagnosis and medical diagnostics in general.

1.7. Aim of the thesis

The aim of this thesis is to discuss and present relevant improvements for the diagnosis of leishmaniasis, an infectious disease that affects both humans and dogs. The historical survey in this chapter presents the main technological advances in VL diagnosis since the 1930s. This study clearly shows an evolution towards serological tests and illustrates the importance of developing specific antigens to detect the presence of VL antibodies in samples accurately. The introduction of recombinant molecules has further accelerated the field of VL diagnosis. The new technologies that used these new targets in sensing applications allow for low-cost, fast, and high-throughput screening (such as ICTs or immunobiosensors) and have offered new tools for screening large populations. The literature study presented, has given us insights in what type of technological requirement antigenic targets and the resulting sensor should have in order to contribute in a meaningful manner to the field.

The need for developing improved diagnostic strategies, and therefore the studies conducted in this thesis, is explicitly emphasized by the fact that despite efforts to control the disease, the incidence of human and canine VL is still high mainly in

low-and middle-income countries. The reason for this is that the currently used diagnostic tests all have their limitations and many of them suffer from low sensitivity as detailed in the first chapter of the thesis. The diagnostic accuracy of these tests is further comprised, especially in endemic areas, because most dogs infected by *Leishmania* are asymptomatic and are not properly diagnosed by serological tests, due to the low levels of antibodies that these dogs can present. Thus, the following chapters approach how dogs have an impact on the spread of the disease mainly asymptomatic dogs. The difficulty of current diagnostic tests to identify asymptomatic dogs is also one of the focuses of this thesis to improve the future design of the technologies presented so that they can also address this major issue that is currently limiting VL diagnosis in everyday practice. All this discussion only reinforces the importance of diagnosis for the control of leishmaniasis, because among the control alternatives applied to reduce the incidence of the disease in dogs and consequently in humans, the early identification of infected animals is one of the most important.

Therefore, the main objective of the thesis is the identification, characterization, and evaluation of new more sensitive and specific molecules to be used as serological markers in traditional platforms of the diagnosis of leishmaniasis such as ELISA and ICTs. In chapter 2 and 3 we will focus on identifying new recombinant targets in the kinesin and dynamin-1-like protein superfamilies as they are hypothesized as potentially interesting candidates for VL diagnosis. In chapter 4, epitopes in these recombinant proteins were identified to construct peptides that could improve antigenicity of the serological tests. The research done in this study was carried out at the Laboratório de Imunologia e Controle de Parasitos (LICP) at the Federal University of Minas Gerais (UFMG), Brazil. The LICP is specialized in the process of identification and characterization of new molecules with applications in diagnostics and vaccines. The work however, is part of a bigger, long-term research project in collaboration with Maastricht University (UM) in which these antigenic targets will be implemented into a biosensor for low-cost, fast and high-throughput screening of samples collected from VL carriers and patients.

In this sense, the work presented in this thesis can be regarded as Phase I of the bigger research project. In parallel, we have already started the next phase of the research project as part of a dual degree project with the Sensor Engineering Department of Maastricht University. The UM PhD candidate has developed a thermal biosensor readout technology during a research stay at LICP. In Phase II of the research project, the targets developed in this thesis will form the receptor layer in a biosensor based on a thermal readout platform that was developed in close collaboration with UM. This thermal biosensor will allow for the point-of-care diagnosis of VL. In comparison to commercial devices currently on the market, the device will offer superior sensitivity over low-cost devices such as lateral flow assays while being much cheaper and faster than high-end laboratory technologies.

2. Chapter II – KDDR-PLUS protein

The increased presence of repetitive motifs in the KDDR-plus recombinant protein, a kinesin-derived antigen from *Leishmania infantum*, improves the diagnostic performance of serological tests for human and canine leishmaniasis.

Adapted from:

Siqueira WF, Viana AG, Reis Cunha JL, Rosa LM, Bueno LL, Bartholomeu DC, Cardoso MS, Fujiwara RT. The increased presence of repetitive motifs in the KDDR-plus recombinant protein, a kinesin-derived antigen from *Leishmania infantum*, improves the diagnostic performance of serological tests for human and canine visceral leishmaniasis. PLoS Negl Trop Dis. 2021 Sep 17;15(9):e0009759. doi: 10.1371/journal.pntd.0009759.

2.1. Chapter context

This chapter focuses on the search for improved markers for the serological diagnosis of human and canine leishmaniasis. In this chapter, kinesins was investigated. They belong to the protein family that includes one of the most commonly used antigens in the serological diagnosis of VL. Seeking to understand the role of this protein superfamily in improving the diagnosis of VL, different compositions of amino acid sequences present in kinesin were compared. The best amino acid sequence for VL diagnosis was defined and it was investigated what makes them preferable over other sequences. The obtained knowledge was applied by using the sequence for the serodiagnosis of VL.

2.2. Chapter abstract

Visceral leishmaniasis (VL) is caused by protozoa belonging to the *Leishmania donovani* complex and is considered the most serious and fatal form among the different types of leishmaniasis, if not early diagnosed and treated. Among the measures of disease control stand out the management of infected dogs and the early diagnosis and appropriate treatment of human cases. Several antigens have been characterized for use in the VL diagnosis, among them the recombinant kinesin-derived antigens from *L. infantum*, such as rK39 and rKDDR. The main difference between these antigens is the size of the non-repetitive kinesin region and the number of repetitions of the 39 amino acid degenerate motif (6.5 and 8.5 repeats in rK39 and rKDDR, respectively). This repetitive region has a high antigenicity score. To evaluate the effect of increasing the number of repeats on diagnostic performance, the rKDDR-plus antigen, containing 15.3 repeats of the 39 amino acids degenerate motif, without the non-repetitive portion from *L. infantum* kinesin was designed. Its performance was evaluated by enzyme-linked immunosorbent assay (ELISA) and rapid immunochromatographic test (ICT), and compared with the kinesin-derived antigens (rKDDR and rK39). In ELISA with human sera, all recombinant antigens had a sensitivity of 98%, whereas the specificity for rKDDR-plus, rKDDR and rK39 was 100%, 96% and 71%, respectively. When evaluated canine sera, the ELISA sensitivity was 97% for all antigens, and the specificity for rKDDR-plus, rKDDR and rK39 was 98%, 91% and 83%, respectively. Evaluation of the ICT/rKDDR-plus, using human sera, showed greater diagnostic sensitivity (90%) and specificity (100%), when compared to the IT LEISH (79% and 98%, respectively), which is based on the rK39 antigen. These results suggest that the increased presence of repetitive motifs in the rKDDR-plus protein improves the diagnostic performance of serological tests by increasing the specificity and accuracy of the diagnosis.

2.3. Introduction

The leishmaniasis is a complex group of infectious parasitic diseases that presents a broad spectrum of clinical manifestations. The visceral form of leishmaniasis, or kala-azar, is considered one of the most lethal and neglected

diseases in the world (5). Visceral leishmaniasis (VL) is endemic in many countries representing a serious public health problem (4). It is estimated 0.2 to 0.4 million cases of VL in the world and 50,000 to 90,000 new cases each year (48, 49). In the Americas and Southern Europe, the VL presents zoonotic character, being the dog its main reservoir in urban areas. In these regions the VL is considered a disease of great human and veterinary medical importance (50). In order to reduce the disease morbidity and lethality rates, the World Health Organization (WHO) recommends the joint use of several control strategies aimed at the main agents involved in this pathology (51). One of the control strategies involves the early and accurate diagnosis for effective treatment (52).

Serological methods are important allies in the diagnostic of the VL, since this disease is characterized by a large production of specific antibodies against parasite antigens and by the stimulation of the polyclonal B cells in hosts (53-55). Serological techniques, such as enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT), are relatively simple, easy to execute, have low-cost and fast result, and can be performed manually or be automated (21, 56). The ICT still has the advantage of being technology and skilled labor independent, making this test an excellent tool for mass screening in the field (21). These techniques allow the use of different types of antigens, such as the crude or soluble extract of *Leishmania* ssp. or recombinant proteins (57). The sensitivity and specificity these techniques vary according to the antigen used (14). Despite the numerous advantages, the serological tests have limitations, such as the detection of asymptomatic dogs and individuals or in early stage of the disease and the occurrence of cross-reactions with related diseases (27). Recombinant molecules have emerged as an alternative to improve the quality of serologic diagnostics (9), with varying sensitivity and specificity (2, 58, 59).

Studies with protozoa show that antigens that have tandem repeat (TR) motifs in their sequence are possible targets for diagnosis, since they are usually highly antigenic B-cell epitopes (60-62). Several TR proteins have also been reported as candidates for serodiagnosis of VL, as rK39 (19), A2 (22), rK26 (23), rKDDR (28), in addition to others identified by serological screening from an expression library of *L. infantum* (60).

One of the main antigens currently used in the diagnosis of VL is the rK39, composed of an immunogenic epitope that presents 6.5 copies of a tandemly arrayed 39 amino acid repeat, encoded by a kinesin-related gene from *L. infantum* (19). The rK39 stands out from the other antigens described in the literature for its high capacity to discriminate patients with VL from healthy patients (9, 19, 63, 64). However, its sensitivity and specificity vary a lot according to the diagnostic method used and depending on the geographic region (65, 66). Recently, a new antigen named rKDDR, kinesin-derived from *L. infantum* and containing 8.5 TR motifs of the 39 amino acids, was evaluated for the diagnosis of VL. The rKDDR showed greater sensitivity and specificity when compared to rK39, both with human and dogs sera (28). The promising relationship of these antigens in the VL diagnosis is probably associated with the presence of these TR regions, which seem to confer a greater antigenic capacity to proteins, being preferentially recognized by the antibodies present in the sera of patients (60, 67).

The influence of the number of TR domains of a protein on antibody reactivity was previously reported by Goto *et al*. (68). These authors showed that the antigenicity of these proteins is possibly influenced by their repetitive composition and by their upregulated expression in *Leishmania* amastigotes, which is the developmental stage in the mammalian host (68). However, the effect of the number of these TR motifs on the diagnostic performance of an antigen remains unclear. Therefore, the objective of this study was to evaluate the impact of the progressive increase in the number of repetitive motifs in a recombinant protein for the diagnosis of VL. For this, the performance of a new kinesin-derived antigen from *L*. *infantum*, named rKDDR-plus, composed of an increased succession of TR sequences of 39 amino acids, totaling 15.3 repetitive blocks was assessed. Comparative serological tests were performed (ELISA and ICT) between the protein rKDDR-plus and the antigens also derived from kinesin, rKDDR and rK39, and the diagnostic efficiency was evaluated.

2.4. Methods

2.4.1. Ethics statement

To use of human sera in this study was submitted and approved by Human Research Ethics Committee (protocol number CAAE—00842112.2.0000.5149) of the Federal University of Minas Gerais. Participating individuals signed an Informed Consent Form agreeing to participate in the study. For patients younger than 18 years of age, the parents signed the Informed Consent Form, consenting to the child's participation. Approval for use of the animal sera was obtained from the Ethics Committee on Animal Use (protocol number 44/2012) of the Federal University of Minas Gerais.

2.4.2. Design and cloning of the KDDR-plus synthetic gene

The KDDR-plus synthetic gene was designed based on the nucleotide sequence of the *L*. *infantum* kinesin gene (LINF_140017300), which was obtained from the TriTrypDB database (http://tritrypdb.org). This gene was synthesized by the GenScript company (USA) in pUC57 cloning plasmid. The KDDR-plus synthetic gene includes the nucleotides 1,198 to 2,985 of *L*. *infantum* kinesin gene, totalizing 1,788 base pairs (bp). This sequence encodes 15.3 repeats of 39 amino acids presents in LINF_140017300 gene. Sites of restriction enzymes *Nhe*I and *Xho*I were added at 5' and 3' ends of the synthetic gene, respectively, to facilitate the subcloning of the gene into the pET28a-TEV expression vector (CeBiME, Campinas/SP, Brazil) and a stop codon (TAA) was added at 3' end. The original codons of synthetic gene were optimized for the protein translation into *Escherichia coli* bacteria used as host cell for heterologous protein expression.

The pUC57/KDDR-plus plasmid was digested with the restriction enzymes to release the synthetic gene that was subsequently subcloning using the restriction sites of the pET28a-TEV plasmid. *E*. *coli* BL21 Star competent cells were transformed with the pET28a-TEV/KDDR-plus recombinant plasmid by electroporation. Correct

gene insertion was confirmed by colony PCR and Sanger automated sequencing, performed by the Macrogen company (Seul, South Korea), using the sequencer ABI Prism 3730xl DNA Analyzer (Applied Biosystems, USA) and the T7 forward and T7 reverse primers to the pET28a-TEV vector.

2.4.3. Heterologous expression and purification of the recombinant protein rKDDR-plus

The expression of rKDDR-plus in transformed *E. coli* cells was induced after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, Brazil) and incubation for 3 h at 37°C at 180 rpm, using the shaker Maxq 400 (Thermo Scientific, USA). After centrifugation, the bacterial cells were resuspended in buffer A (20 mM NaH_2PO_4; 500 mM NaCl; 30 mM imidazole) in 10% of the initial volume of the culture. The cells were lysed with lysozyme (100 µg/mL) for 30 min followed of 5 cycles of mechanical lysis in the EmulsiFlex-C3 homogenizer (AVESTIN, Canada), using pressure peaks between 15,000 to 20,000 psi. The suspension was centrifuged at 6,000x*g* for 1 h at 4°C. Samples referring to the soluble and insoluble fraction were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (69) for determined the solubility of the recombinant protein. Fractions of the cell extract before (0 h) and after (3 h) the addition of IPTG were also analyzed by SDS-PAGE.

The recombinant protein was purified by Ni^{2+} affinity chromatography, using the His-Trap HP column (GE Healthcare Life Science, Brazil) coupled to an ÄKTA Prime Plus system (GE Healthcare Life Science, Brazil). The rKDDR-plus protein was eluted with a linear gradient from 0 to 100% of buffer B (NaH_2PO_4 20 mM; NaCl 500 mM; imidazole 500 mM) in a volume of 10 mL. To assess the purity and yield of the purified recombinant protein, the fractions obtained were analyzed by SDS-PAGE and the final concentration was indirectly determined by the BCA Protein Assay (Thermo Fisher Scientific, USA), according to manufacturer's instructions.

2.4.4. Bioinformatics analyses

The amino acid sequence of the rKDDR-plus protein was submitted to the BepiPred program (http://www.cbs.dtu.dk/services/BepiPred-1.0), with a cut-off of 1.0 (sensitivity < 0.25; specificity > 0.91), to prediction of linear epitopes of B-cells (70), and IUPred program (http://iupred.elte.hu/), with cut-off of 0.5 (score range of 0.0–1.0), to prediction of the protein structural disorder (71). The theoretical molecular weight and isoelectric point (pI) were analyzed through the ProtParam program (https://web.expasy.org/protparam/).

2.4.5. Serum Samples

Human sera

Sera of 126 individuals were used in this study: 50 samples from patients with human VL (HVL) obtained from the University Hospital Clemente of Faria (Montes Claros, Minas Gerais State, Brazil), endemic region located in southeastern Brazil. The *L. infantum* infection was determined, from bone marrow samples, by the parasitological method (detection of the parasite) using Giemsa stain, and by specific qPCR assays for kDNA from the *Leishmania* parasite (72). Samples from 54 patients chronically infected with *Trypanosoma cruzi* (Tc), to evaluate cross reactivity, were obtained from the University Hospital Clemente of Faria (Montes Claros, Minas Gerais State, Brazil) with infection confirmed by combination of positivity in Chagatest recombinant ELISA v.3.0 kit (Wiener Lab, Argentina) and the Chagatest Indirect Hemagglutination Assay (IHA) (Wiener Lab, Argentina). In addition, 22 sera from healthy individuals were used as negative control (NC) from an endemic area for VL and with negative results for *Leishmania* in tissue smears (bone marrow).

Canine sera

A total of 180 canine sera were used in the study. Sixty serum samples were collected in an endemic region for canine (CanL) (Montes Claros, Minas Gerais State, Brazil), and 36 samples from healthy dogs (not infected) were used as non-endemic controls (NC), both confirmed by microscopic analysis of bone marrow aspirates. For cross-reactivity assessment of the tests were used 48 sera from dogs infected with *T. cruzi* (Tc), 27 dogs infected with *Babesia* sp. (Bab) and 9 with *Ehrlichia* sp. (Ehr). Serum samples from *T. cruzi* were obtained from Department of Clinical Analysis of the School of Pharmacy/UFOP. Samples from dogs infected with *Babesia* sp. and *Ehrlichia* sp. were obtained from a private veterinary laboratory (Contagem/Minas Gerais State, Brazil) and positivity to both the parasites was confirmed by parasitological techniques (blood smears).

2.4.6. Crude soluble antigen of *L. infantum*

The *L. infantum* reference strain MHOM/BR/1974/PP75 was used in this study. *L. infantum* promastigotes were cultured at 24°C in Schneider's medium (Sigma-Aldrich, USA), supplemented with 10% of inactivated bovine fetal sera, containing 100 U/mL de penicillin and 100 µg/mL de streptomycin (Gibco/Thermo Fisher Scientific, USA). Approximately $5x10^8$ parasites were washed and recovered in 1 mL de PBS followed of 15 lysis cycles of freezing in liquid nitrogen and thawing at 37°C.

2.4.7. Enzyme-linked immunosorbent assay (ELISA)

The performance of the rKDDR-plus, rKDDR (28), rK39 (19) and rK26 (23) proteins and crude soluble antigen (CSA) of *L. infantum* was evaluated by ELISA. The rKDDR protein was obtained and purified as described by Dhom-Lemos *et al*. (28). The rK39 and rK26 antigens were kindly provided by Steven G. Reed (Infectious Disease Research Institute—IDRI, Seattle, Washington). The optimal coating concentrations of the recombinant antigens used in this work were determined empirically through a titration optimization using these proteins. The concentration of CSA was performed as previously described (19). All antigens were diluted in 100 µL carbonate buffer [Na_2CO_3 15 mM (Synth, Brazil); $NaHCO_3$ 34 mM (Merck, Brazil) pH 9.6] in amounts of 50 ng per well to the rKDDR-plus and rKDDR proteins, and 100 ng per well to the rK39 and rK26 proteins and CSA. ELISA microplates of 96 wells (Costar, USA) were coated with the antigens and incubated for 16 h at 4°C. To avoid non-specific links, the plate was blocked with 2% BSA (Fitzgerald, USA) in PBS pH 7.2 for a period of 2 h at room temperature. Human and canine serum samples diluted 1:100 in PBS containing 0.05% Tween 20 (PBS-T) were added (100 µL/well) and incubated for 16 h at 4°C. Plates were washed in wash buffer (PBS-T) five times and incubated with 100 µL of the anti-human IgG (Fc specific) − peroxidase antibody produced in goat (#A0170, Sigma-Aldrich, USA) or anti-dog IgG (whole molecule)–peroxidase antibody produced in rabbit (#A6792, Sigma-Aldrich, USA), diluted in PBS-T at 1:5,000 to human sera or 1:2,500 to canine sera, and incubated at 37°C for 1 h and 30 min. After a final wash step of the plates, the enzyme reaction was developed with 100 µL/well of substrate solution [0.1 M citric acid, 0.2 M Na_2PO_4, 0.05% *o*-phenylenediamine dihydrochloride (OPD) (Sigma- Aldrich, USA) and 0.1% H_2O_2] for 10 min at 37°C. The reaction was stopped using 50 µL of 4N sulfuric acid (H_2SO_4) and the plates were read at 492 nm in an automated microplate ELISA reader (Versamax, Molecular Devices, USA).

2.4.8. Rapid immunochromatographic dipstick test (ICT)

Development of the ICT/KDDR-plus

Figure 1 shows the ICT prototype developed in partnership with Safetest Diagnósticos (Brazil) based on the rKDDR-plus protein. The device is formed by an initial region containing a sample application pad, which adsorbs the sample and distributes it homogeneously to the membrane, followed by a conjugate release membrane, in which there are colloidal gold nanoparticles, colored in red, linked to the rKDDR-plus antigen or the mouse IgG antibody. Soon after, the rKDDR-plus antigen is immobilized on the membrane within the test area, followed by the control area, in which there is a capture reagent (anti-mouse IgG), which acts as a validation of the test reactivity. After the control line there is an absorbent pad that decreases the background color by increasing the sample flow volume. The device was stored in the dark in aluminum bags containing silica at room temperature until use.

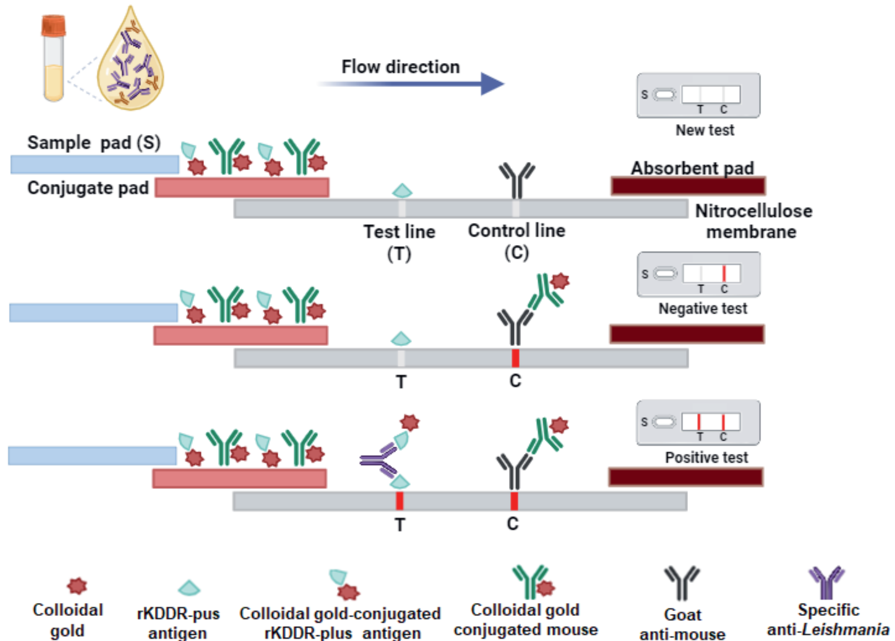

Figure 1 - Schematic representation of the diagnostic ICT device. The three schematic bands represent the test steps: a new test, a negative test and a positive test.

ICT/rKDDR-plus for HVL diagnosis

Assays to detect anti-leishmanial antibodies were carried out with ICT/KDDR-plus prototype, using 69 human sera randomly selected from the serological panel previously described, being 29 serum samples from patients infected with *Leishmania* (HVL); 20 from patients chronically infected with *T. cruzi* (Tc); and 20 from healthy individuals (NC). The performance of the ICT/rKDDR-plus (Safetest Diagnósticos, Brazil) was evaluated and compared to IT LEISH ICT by DiaMed AG (Cressier, Switzerland) and distributed by DiaMed Latin America S.A. (Lagoa Santa, Brazil), based on the rK39 antigen, following the manufacturer instructions. The evaluation of the test was performed in duplicate and the qualitative analysis of the results was performed by the naked eye 20 min after the beginning of the test by two independent evaluators. The results were established by the presence (positive result) or absence (negative result) of a red line in the test area, under the condition that a red line could be visualized in the control area as exemplified in figure 2.

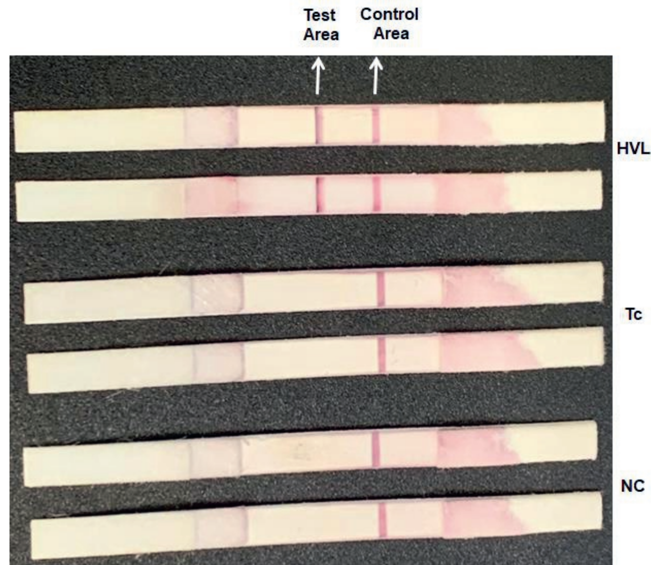

Figure 2 - Rapid immunochromatographic lateral flow test (ICT) prototype using the rKDDR-plus antigen. Photograph of representative results of ICT/rKDDR-plus strips tested with different human serum samples. HVL: test with sera from two patients infected with *Leishmania*; Tc: test with sera from two patients infected with *T. cruzi*; NC: test with two negative sera from healthy individuals. With negative serum samples for leishmaniasis (NC and Tc), only the control line was present, while with serum samples from VL patients, both control and test lines turned red.

2.4.9. Data analysis

The ELISA data were analyzed using the Software GraphPad Prism 5.0 (GraphPad Prism Inc., USA) and MedCalc with confidence intervals of 95%. Through the receiver, operating characteristic (ROC) curve was possible to evaluate all the combinations of the sensibility and specificity and to determine a cut-off value for each antigen tested. For the ROC curve analysis, the infected group was composed of VL samples and the control group was composed of non-VL samples. Area under the curve (AUC) was calculated and diagnostic performance was established. The positive predictive values (PPV) and negative predictive values (NPV) as well as the accuracy (AC) of the tests were calculated by the ratio of true positive (TP) and false-negative (FN). The degree of agreement between the serological tests (ELISA and ICT) and the parasitological test (biopsy, aspirate or PCR) was estimated by kappa index (k) with 95% confidence interval and classified according to the Fleiss scale: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; 0.81–0.99, very good; and 1.00, perfect.

2.5. Results

2.5.1. Composition and molecular analysis of the rKDDR-plus

The complete nucleotide sequence of the *L. infantum* kinesin gene (LINF_140017300) has 10,830 bp and encodes a protein with 3,609 amino acids, divided into two different portions: a non-repetitive amino acid sequence at the amino-terminal region of the protein and a repetitive region containing a tandem block of 39 amino acids, with some degenerations in the sequence. The KDDR-plus synthetic gene was designed based on the sequence encoding 15.3 tandem repeats of 39 amino acids present in the kinesin protein, totalizing 1,788 nucleotides.

The rKDDR-plus protein was formed only by the repetitive portion of *L. infantum* kinesin protein, unlike other proteins derived from kinesin and widely used in the diagnosis of VL in the world, such as rKDDR (28) and rK39 (19) (Figure 3). In addition, this antigen has a higher number of motif repetitions of 39 amino acids (15.3 repeats), when compared with the other proteins derived from kinesin: rK39 (6.5 repeats) and rKDDR (8.5 repeats).

Figure 3 - Comparison of the structure of the rKDDR-plus and other antigens based on the kinesin protein of *L. infantum*.

The kinesin-derived proteins used in the comparison were rKDDR-plus, rKDDR, and rK39. The red numbers represent the amount of amino acids present in each block and the black numbers indicate the position of the block. The green blocks represent the non-repetitive portion of amino acids present in the kinesin proteins. The gray blocks represent the repetitive motif units of 39 amino acids present in each protein. The blue blocks indicate repetitive blocks present in the kinesin protein, but which have variations of certain amino acids in relation to the consensus sequence of 39 amino acids described by Burns *et al.* (1993). The orange blocks represent the incomplete repetitive motif units of amino acids.

The synthetic KDDR-plus gene had optimized *in silico* codons, and then cloned into the pET28a-TEV expression vector, which contains a sequence encoding a histidine tag. After cloning, a single ORF (Open Reading Frame) with 1,848 bp was obtained, which encodes 615 amino acids (Figure 4), with a theoretical molecular mass of 68 kDa and a pI of 4.56. Thus, 97% (596 amino acids) of the protein sequence

consists exclusively of repetitive motifs, while the remaining 3% (19 amino acids) originate from the plasmid sequence.

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       atgggccatcatcatcatcatcacgaaaacctgtattttcagggccatatggctagcaagagcagcgcggagcaggaccgtgaaaacacccgtgcgacc   99
1°      M  G  H  H  H  H  H  H  E  N  L  Y  F  Q  G  H  M  A  S  K  S  S  A  E  Q  D  R  E  N  T  R  A  T
    ctggagcagcaactgcgtgagagcgaagcgcgtgcggcggagctggcgagccagctggaagcgaccgcggcggcgaaaatgagcgcggagcaagatcgcgagaatacccgtgcgacc   216
2°  L  E  Q  Q  L  R  E  S  E  A  R  A  A  E  L  A  S  Q  L  E  A  T  A  A  A  K  M  S  A  E  Q  D  R  E  N  T  R  A  T
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3°  L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  S  T  R  A  T
    ctggagcaacagctgcgcgacagcgaggaacgcgctgccgagctggcgagccagctggaatctactaccgctgctaagatgagcgcggaacaggatcgtgaatcgacccgtgcgact   450
4°  L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  S  T  R  A  T
    ctggagcagcagctgcgcgaaagcgaggaacgcgctgcagagctggcgagccagctggaaagtactaccgctgctaaaatgagcgcggaacaggaccgtgaaagtacccgtgcgact   567
5°  L  E  Q  Q  L  R  E  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  S  T  R  A  T
    ctggaacaacagctgcgcgatagcgaagaacgcgctgcggagctggcgagccaactggaagctactgcggcggcgaaaagcagcgcggaacaggatcgcgaaaacacccgtgcggcg   684
6°  L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  A  T  A  A  A  K  S  S  A  E  Q  D  R  E  N  T  R  A  A
    ctggagcaacagctgcgtgacagcgaagaacgcgccgctgagctggcgagccagctggaaagcactactgctgccaagatgagcgcggaacaagatcgtgaatccacccgtgcgact   801
7°  L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  S  T  R  A  T
    ctggaacagcagctgcgcgatagcgaggaacgcgccgccgagctggcgagccagctggaaagtacgaccgctgcaaagatgagcgcggagcaggatcgtgagaatacccgtgcgact   918
8°  L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  S  T  R  A  T
    ctggagcagcaactgcgcgacagcgaagagcgcgccgcagagctggcgagccagctggaagcaaccgcggctgccaagatgtctgctgaacaagatcgcgagaacacccgtgcggct  1035
9°  L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  A  T  A  A  A  K  M  S  A  E  Q  D  R  E  N  T  R  A  A
    ctggaacaacaactgcgcgacagcgaggagcgcgccgcggagctggcgagccagctggaagctaccgcggcggcgaagagcagcgcggaacaggaccgcgaaaatacccgtgcggct  1152
10° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  A  T  A  A  A  K  S  S  A  E  Q  D  R  E  N  T  R  A  A
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11° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  N  T  R  A  T
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12° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  A  T  A  A  A  K  M  S  A  E  Q  D  R  E  N  T  R  A  A
    ctggagcaacagttacgcgacagcgaggaacgagctgcagagctggcgagccagctggaaagtacaaccgctgccaaaatgagtgcggaacaagatcgcgaatcaacccgtgcgaca  1503
13° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  S  T  R  A  T
    ctggagcaacagttacgagacagcgaggaacgcgcagcagagctggcgagccagctggaaagtacgacggctgccaagatgagcgctgaacaagatcgtgaaaatacccgtgcgaca  1620
14° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  N  T  R  A  T
    ctggagcaacagcttcgcgatagcgaggaacgagccgcagagctggcgagccagctggaagcaacggcggctgcaaagatgtctgctgaacaggatcgagagaatacccgtgcggca  1737
15° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  S  T  T  A  A  K  M  S  A  E  Q  D  R  E  N  T  R  A  A
    ctggagcaacagctccgcgatagcgaggaacgggctgcagagctggcgagccagctggaagcaactgctgctgcgaagagcagcgcggagcaagatcgtgagaacacctaactcgag  1854
16° L  E  Q  Q  L  R  D  S  E  E  R  A  A  E  L  A  S  Q  L  E  A  T  A  A  A  K  S  S  A  E  Q  D  R  E  N  T  -
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Figure 4 - Schematic representation of the DNA sequence and predicted amino acid sequence of the rKDDR-plus protein. Lowercase letters represent the nucleotide sequence of KDDR-plus and uppercase letters represent the translated protein sequence. The nucleotides and amino acids indicated in red at the ends of the sequences represent the portion derived from the plasmid pET28a-TEV, used in the expression of the protein in bacteria. The underlined nucleotides in purple correspond to the initiation codon; the underlined in yellow represent the histidine tag added to the protein to facilitate the purification process; the underlined nucleotides in blue correspond to the sites for the restriction enzymes; and the underlined in green represent the cleavage site for the TEV protease. The remainder of the underlined sequence shows the 15.3 repetitive motifs of 39 amino acids derived from the *L. infantum* kinesin protein, except for the first motif with 14 amino acids and the 16th motif with 36 amino acids. The ordinal numbers indicated on the left represent the number of repetitive motifs of rKDDR-plus. The numbers on the right represent the position of the nucleotides.

The construct pET28a-TEV/KDDR-plus was transformed into *E. coli* BL21 Star and the recombinant rKDDR-plus protein was expressed by IPTG induction and presented a high yield after 3 h of induction. The solubility test indicated that the protein was soluble and could be purified under native conditions as seen in Figure 5 A. After purification by affinity chromatography, the rKDDR-plus protein was obtained with a high level of purity. Analysis of the purified fraction revealed the presence of a highly expressed 68 kDa band, referring to rKDDR-plus as shown in Figure 5 B, with an estimated yield of approximately 3 mg of protein per 1 L bacterial culture.

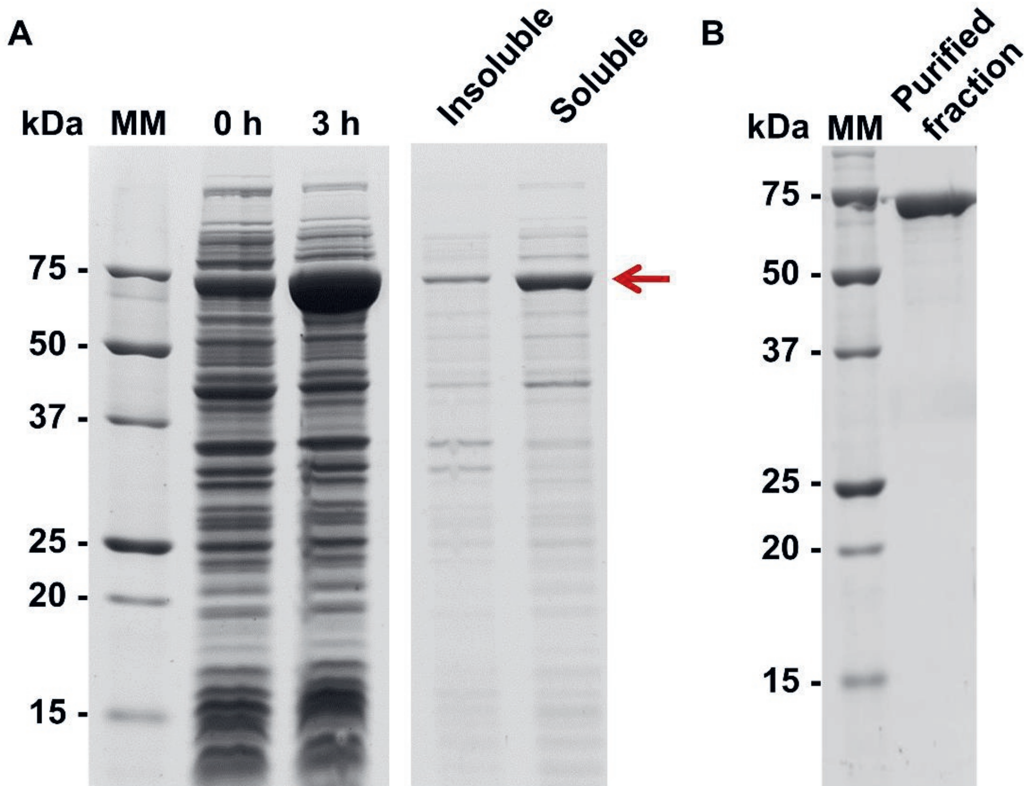

Figure 5 - Analysis in polyacrylamide gel electrophoresis (SDS-PAGE) of the expression in bacteria and purification of rKDDR-plus protein. (A) Extracts of *E. coli* bacteria, BL21 Star strain, containing the plasmid pET28a-TEV/KDDR-plus, before (0h) and after (3h) induction of the recombinant protein with IPTG (1 mM). The bacterial extract was lysed and separated into soluble and insoluble fraction by centrifugation. The red arrow indicates the rKDDR-plus protein band after expression and solubility test. (B) After purification by affinity chromatography of the soluble fraction of the bacterial lysate, the purified fraction presented a band of approximately 68 kDa, corresponding to rKDDR-plus. MM: molecular mass marker; kDa: kilodalton.

2.5.2. Prediction of linear B-cell epitopes and structural disorder

To evaluate the antigenic potential of rKDDR-plus, the linear B-cell epitopes present in the protein were predicted using the BepiPred 1.0 tool. In addition, the degree of structural disorder along the rKDDR-plus protein was determined using the IUPred tool in order to verify regions with no secondary structures.

Figure 6 shows the data obtained in bioinformatics analysis indicating the presence of a large number of linear B-cell epitopes in rKDDR-plus, suggesting that this protein is probably capable of interacting with lymphocytes of the host immune system and can be applied as a possible target for the VL diagnosis. The presence of a large region of structural disorder was also observed along the length of the rKDDR-

plus protein sequence. These results suggest that the B-cell epitopes present in the protein were available to be recognized by the host immune system since they were not involved in internal secondary interactions within the protein.

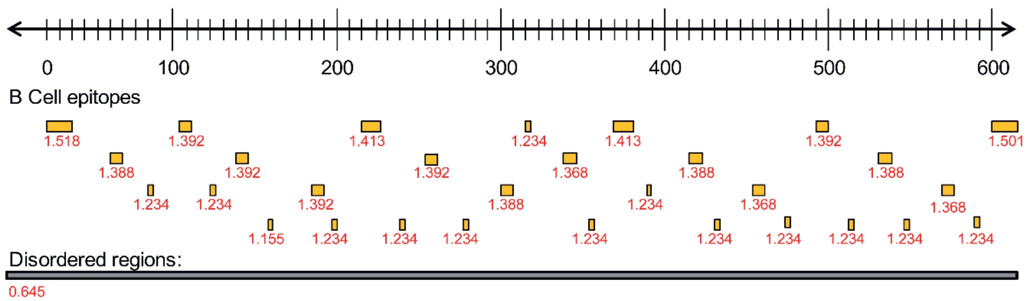

Figure 6 - Predictions of linear B-cell epitopes and structural disorder regions of rKDDR-plus protein. The dashed arrow corresponds to the complete amino acid sequence of the protein. The orange boxes correspond to the linear B-cell epitopes predicted by the BepiPred program, while the gray box corresponds to the prediction of structural protein disorder using the IUPred program. The value below each box corresponds to the score of each prediction.

2.5.3. ELISA tests with rKDDR-plus for detection of IgG antibodies in patients with human visceral leishmaniasis

The recognition potential of the rKDDR-plus antigen for the HVL diagnosis was determinate by assessing the reactivity of this protein in ELISA followed by the comparison of its performance with the antigens also derived from kinesin, rKDDR (28) and rK39 (19), and also with the rK26 protein (23) and the CSA, used as controls as presented in Figure 7. The rK26 antigen is derived from the hydrophilic surface protein B, which contains 11 repeats of 14 amino acids that comprise 64% of the total protein (23). This protein was used as a control, as it is a repetitive protein not derived from kinesin.

The rKDDR-plus, rKDDR and rK39 antigens showed the same percentage of serum recognition from patients with HVL (98%) (Figure 7 A). In turn, rK26 and CSA showed 82% and 100% recognition of the positive sera, respectively. However, rKDDR-plus was the only antigen that did not cross-reaction with sera from chagasic patients compared to the other antigens evaluated. In addition, the rK39 and rK26 antigens also identified sera from control group individuals (not infected) as positive. The cut-off of ELISA plots was determined for each antigen from the ROC curve (Figure 7 B). The cut-off for the rKDDR-plus, rKDDR, rK39 and rK26 antigens and the CSA was 0.3611, 0.4437, 0.5839, 0.3977 and 1.284 absorbance units (AU), respectively.

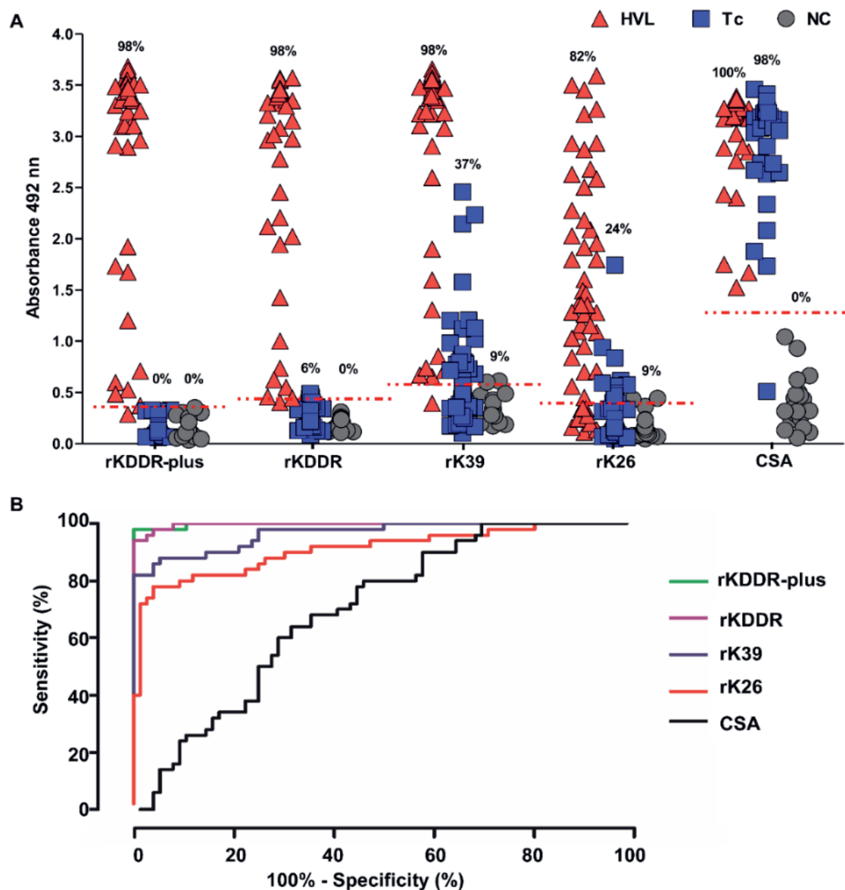

Figure 7 - Evaluation of the recombinant antigens and *L. infantum* crude extract for the serodiagnosis of human visceral leishmaniasis (HVL). (A) Comparison of the performance of ELISA with the rKDDR-plus, rKDDR, rK39 and rK26 antigens and the crude soluble antigen (CSA) of *L. infantum* was performed using serum samples from the following groups: patients infected with *Leishmania* (HVL, n = 50); healthy individuals used as negative control (NC, n = 22); and patients chronically infected with *T. cruzi* (Tc, n = 54), to evaluate cross reactivity. The dashed red line represents the cut-off determined by the ROC curve of each test. The index above each column in the plot indicates the percentage of samples that were above the cut-off. (B) Analysis of ROC curve of each human serological test obtained for ELISA using rKDDR-plus, rKDDR, rK39 and rK26 antigens and the CSA. The sensitivity, specificity and area under the curve (AUC) were determined by ROC curve.

Table 3 shows the general performance of the rKDDR-plus protein and the other analyzed proteins. Analysis of the AUC values using ROC curves confirmed the better performance of the rKDDR-plus antigen (AUC = 0.9979), suggesting that this protein presents a great potential to discriminate the cases of patients with and without the HVL. According to the Fleiss scale, the degree of agreement of rKDDR-plus (k =

0.983), with the parasitological/qPCR reference test, was classified as “very good”, suggesting to be a diagnostic test of excellent quality. The sensitivity, specificity, positive and negative predictive value, as well as accuracy was determined for the five antigens tested. The rKDDR-plus, rKDDR and rK39 antigens had the same sensitivity (98%), while rK26 and the CSA showed 82% and 100%, respectively. However, the rKDDR-plus ELISA showed the best results of specificity (100%), PPV (100%), NPV (98.7%) and accuracy (99.2%), when compared to other antigens that presented lower values.

Table 3 - Diagnostic performance of ELISA tests using human serum samples from patients with visceral leishmaniasis (VL) and non-VL individuals.

Antigen	PPV (%)	NPV (%)	Sen. (%) [CI 95%]	Spe. (%) [CI 95%]	AC (%)	AUC	Kappa [CI 95%]	Agreement
rKDDR-plus	100	98.70	98.0 [89.4 – 100]	100 [95.3 - 100]	99.2	0.998	0.983 [95.1 - 100]	Very good
rKDDR	94.2	98.7	98.0 [89.4-100]	96.1 [88.9 - 99.2]	96.8	0.997	0.934 [87.1 - 99.8]	Very good
rK39	69.0	98.2	98.0 [89.4 – 100]	71.1 [59.5 - 80.9]	81.8	0.966	0.644 [52.0 - 76.9]	Good
rK26	73.2	87.1	82.0 [68.6 - 91.4]	80.3 [69.5 - 88.5]	81.0	0.910	0.610 [47.1 - 75.0]	Good
CSA	48.0	92.3	100.0 [92.9 - 100]	30.3 [20.3 - 41.9]	57.1	0.698	0.236 [12.5 - 34.6]	Fair

PPV: positive predictive value, NPV: negative predictive value, Sen.: sensitivity, Spe.: specificity, AC: accuracy, AUC: area under curve, CI: confidence interval, CSA: crude soluble antigen. Reference test: parasitological/qPCR

2.5.4. Serological recognition of rKDDR-plus by ELISA for the diagnosis of canine leishmaniasis

All the antigens tested by ELISA had the same percentage of serological recognition (97%) of sera from dogs with CanL, except rK26 (92%) (Figure 8 A). However, rKDDR-plus presented a lower percentage of cross-reaction between the tested antigens, only 11% with babesiosis. For other sera used as control, this antigen showed no cross-reactivity. The other antigens showed a high percentage of cross-reactivity with babesiosis, Chagas disease and ehrlichiosis. The rK39 and rK26 antigens also showed a reaction with sera from healthy dogs (negative control). The ROC curve was used to determine the cut-off of each antigen, obtaining the following values in absorbance units (AU): 0.3147 (rKDDR-plus), 0.3248 (rKDDR), 0.4087 (rK39), 0.3251 (rK26) and 0.8314 (CSA) (Figure 8 B).

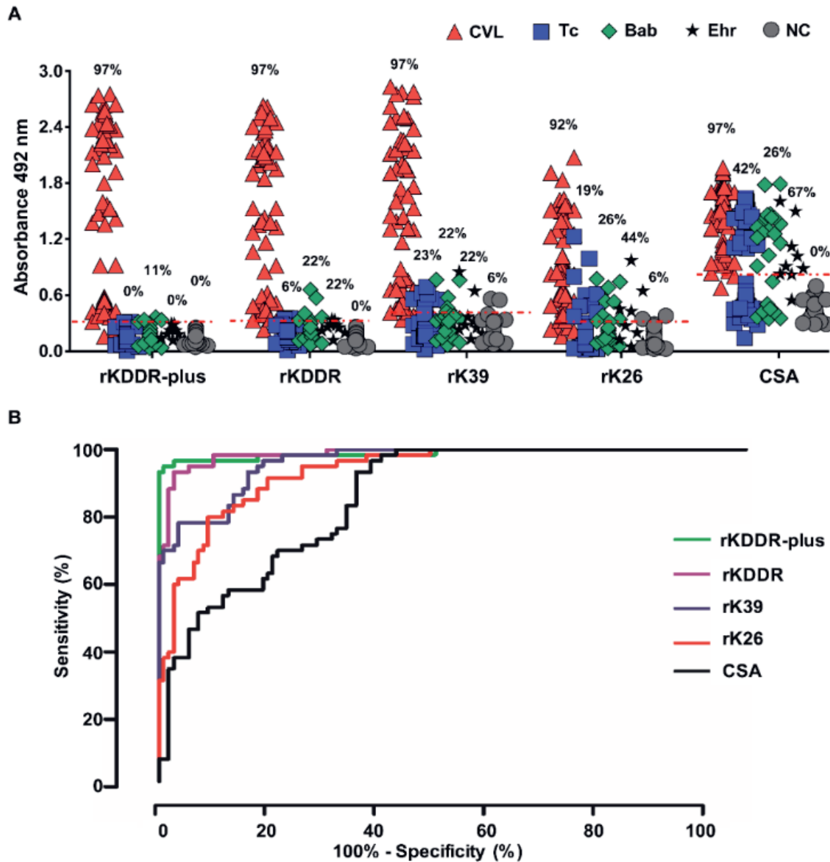

Figure 8 - Evaluation of the recombinant antigens and *L. infantum* crude extract for the serodiagnosis of canine leishmaniasis (CanL). (A) Comparison of the performance of ELISA with the rKDDR-plus, rKDDR, rK39 and rK26 antigens and the crude soluble antigen (CSA) was performed using canine sera from the following groups: dogs infected with *Leishmania* (CanL, n = 60); non-infected healthy dogs (NC, n = 36); dogs infected with *Babesia* sp. (Bab, n = 27) and *Ehrlichia* sp. (Ehr, n = 9); and dogs infected with *T. cruzi* (Tc, n = 48), for cross-reactivity assessment. The dashed red line represents the cut-off determined by the ROC curve of each test. The index above each column in the plot indicates the percentage of samples that were above the cut-off. (B) Analysis of ROC curve of each canine serological test obtained for ELISA using rKDDR-plus, rKDDR, rK39 and rK26 antigens and the CSA. The sensitivity, specificity and area under the curve (AUC) were determined by ROC curve.

Table 4 summarizes the diagnostic performance of the evaluated proteins. The rKDDR-plus showed the highest AUC value (0.9889), followed by rKDDR, rK39, rK26 and the CSA, which presented the values 0.9871, 0.9631, 0.9358 and 0.8608, respectively. All recombinant antigens showed the same sensitivity (96.67%), except

rK26 (91.7%). However, rKDDR-plus has a higher specificity (97.5%) when compared to the rKDDR (90.8%), rK39 (82.5%), rK26 (81.7%) antigens and the CSA (64.2%). The rKDDR-plus also obtained higher PPV (95.1%), NPV (98.3%) and AC (97.2%) compared to the other antigens. The highest kappa concordance index was obtained again by rKDDR-plus (0.938), showing a "very good" degree of agreement, suggesting its potential use for canine serodiagnosis.

Table 4 - Diagnostic performance of ELISA tests to detect antibodies against canine leishmaniasis (CanL).

Antigen	PPV (%)	NPV (%)	Sen (%) [CI 95%]	Spe (%) [CI 95%]	AC (%)	AUC	Kappa [CI 95%]	Agreement
rKDDR-plus	95.1	98.3	96.7 [88.4 - 99.6]	97.5 [92.9 - 99.5]	97.2	0.989	0.938 [88.4 - 99.2]	Very good
rKDDR	84.1	98.2	96.7 [88.4 - 99.6]	90.83 [84.2 - 95.3]	92.8	0.987	0.843 [76.2 - 92.5]	Very good
rK39	73.4	98.0	96.7 [88.4 - 99.6]	82.50 [74.5 - 88.8]	87.2	0.963	0.734 [63.4 - 83.3]	Good
rK26	71.4	95.2	91.6 [81.6 - 97.2]	81.7 [73.6 - 88.1]	85.0	0.934	0.685 [57.8 - 79.2]	Good
CSA	57.43	97.5	96.6 [88.4 - 99.6]	64.2 [54.9 - 72.7]	75.0	0.860	0.520 [41.0 - 62.9]	Moderate

PPV: positive predictive value, NPV: negative predictive value, Sen.: sensitivity, Spe.: specificity, AC: accuracy, AUC: area under curve, CI: confidence interval, CSA: crude soluble antigen. Reference test: parasitological.

2.5.5. Evaluation of ICT/rKDDR-plus for the diagnosis of human visceral leishmaniasis

The ICT based on the rKDDR-plus recombinant antigen (Safetest Diagnósticos, Brazil) had its diagnostic performance evaluated and compared to the IT LEISH ICT, produced by DiaMed AG (Cressier, Switzerland) as seen in Figure 9. Both diagnostic tests were assessed with 69 human serum samples. The ICT/rKDDR-plus detected 26 of 29 sera tested from patients infected with *L. infantum*, resulting in a sensitivity of 89.7%. No false positive result was attributed to ICT/rKDDR-plus, resulting in a specificity of 100%. The accuracy, PPV and NPV of ICT/KDDR-plus were of 95.7%, 100.0% and 93.0%, respectively. In comparison, IT LEISH test identified only 23 of the 29 sera tested from patients with HVL, representing a sensitivity of 79.3%. All individuals of the control group (uninfected) were correctly diagnosed, but when sera from patients with Chagas disease were analyzed, the IT LEISH rapid test identified 1 of 20 sera tested.

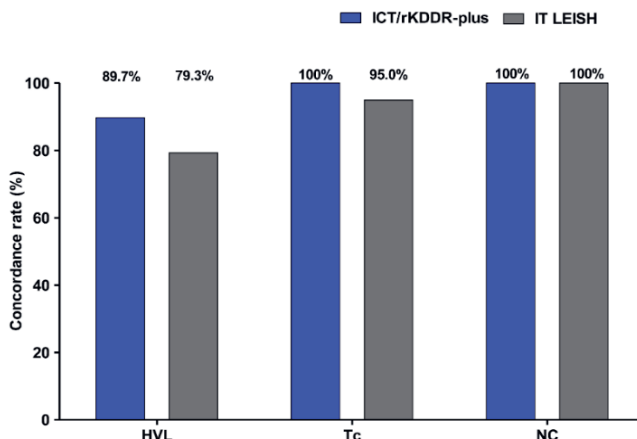

Figure 9 - The concordance rate of the rapid immunochromatographic test results with the reference standard for diagnosis of human visceral leishmaniasis (HVL) with the serum samples. The comparison of the performance between the rapid tests ICT/rKDDR-plus (Safetest Diagnósticos) and IT LEISH (DiaMed AG) was performed using 69 human serum samples from patients infected with *Leishmania* (HVL, n = 29); patients chronically infected with *T. cruzi* (Tc, n = 20), to evaluate cross-reactivity; and healthy individuals used as negative control (NC, n = 20). Reference test: parasitological/qPCR.

Table 5 displays the general diagnostic performance of rapid tests ICT/rKDDR-plus and IT LEISH. These results represent a specificity of 97.5%, with accuracy, PPV and NPV of 89.9%, 95.8% and 86.7%, respectively. The accuracy obtained by IT LEISH against the kappa agreement criterion was considered "good" at kappa index of 0.787, whereas ICT/rKDDR-plus was considered "very good", obtaining a kappa index of 0.909.

Table 5 - Diagnostic performance of immunochromatographic tests with human sera from patients infected with *Leishmania infantum*, Trypanosoma cruzi and healthy controls.

Test	Positive samples	Sen. (%)	**NPV (%)**	**Negative samples**	**Spe. (%)**	**PPV (%)**	**AC (%)**	**Kappa [CI 95%]**	**Agreement**
ICT/ rKDDR -plus	26/29	89.7	93.0	40/40	100	100	95.7	0.909 [81.0-100]	Very good
IT LEISH	23/29	79.3	86.7	39/40	97.5	95.8	89.9	0.787 [63.9-93.5]	Good

PPV: positive predictive value; NPV: negative predictive value; Sen.: sensitivity; Spe.: specificity; AC: accuracy; CI: confidence interval. Reference test: parasitological/qPCR.

2.6. Discussion

In this study, the performance of a new recombinant antigen rKDDR-plus was evaluated. This antigen is composed of an increased number of the repetitive motif of 39 amino acids, present in the *L. infantum* kinesin protein, totaling 15.3 copies. The performance of this new antigen was compared with two other kinesin-derived recombinant antigens described in the literature, rK39 (19) and rKDDR (28), which have a smaller number of repetitive motifs (6.5 and 8.5 copies, respectively), in addition to being also composed of a non-repetitive region of the kinesin protein. In contrast, the rKDDR-plus protein has only the repetitive region of the kinesin protein. The use of TR proteins in the diagnosis of VL was studied by Goto *et al*. (60). In another study, Goto *et al*. (68) performed ELISAs using *Leishmania* recombinant TR proteins and demonstrated that the copy number of the repeat affects the binding affinity between antigens and antibodies, due to thermodynamic binding kinetics. However, the influence of the number of repetitions of a sequence on the diagnostic performance has not yet been evaluated.

One of the current challenges for the VL control revolves around improving the performance of diagnostic tests for human and veterinary use. Several studies have focused on the development of more accurate serodiagnosis, through replacing CSA by recombinant proteins (9, 73-77). Until the early 1990s, the targets used in the serological tests were the crude or soluble antigens of promastigotes or amastigotes forms of *Leishmania* ssp., which has a high sensitivity for the detection of specific antibodies in human and canine sera, but with a very limited specificity (11, 78). Fraga *et al*. (79) showed that the CSA in the ELISA test showed a sensitivity of 97%, but a very limited specificity, 26%. Likewise, another study also showed good sensitivity (100%) when using CSA, but low specificity (68%) (80). Corroborating these results, low specificity was found for both human sera (30.3%) and canine sera (64.2%) in ELISA using CSA.

Recombinant antigens appear as an alternative to the use of CSA, because they are specific molecules of the parasite that have high affinity with antibodies present in the vast majority of biological samples and, unlike the CSA have an excellent specificity (19, 75). In addition to improving the specificity of the tests, without compromising their sensitivity, recombinant proteins allow large-scale and standardized production, independent of parasite cultures, with high efficiency and remarkable purity at relatively low costs (73, 74). Several recombinant molecules have been characterized and evaluated for use with samples from humans and dogs in the VL diagnosis. Among them, it is worth mentioning the proteins with TR motifs in their sequence, as the kinesin-derived antigens of *L. infantum* (rK39 (19) and rKDDR (28)) and the hydrophilic acylated surface protein (rK26) (23).

Regions with successive copies of the same amino acid sequence have been reported in several eukaryotic proteomes, including protozoa such as *Plasmodium* spp., *Trypanosoma* spp. and *Leishmania* spp. (60, 62, 81, 82). Some studies have shown that individuals with VL or other infections, such as trypanosomiasis and malaria, have high production of specific antibodies against these repetitive regions (83-86). Goto *et al*. (60) showed that TR proteins were more potent as immunodominant B-cell antigens, being more highly recognized by VL

patient sera than non-TR proteins. Another study shows that among the reasons for TR proteins to be more antigenic may be related to the exposure of several identical sequences with a single epitope to the host's immune system, generating an immunodominance (60, 87). After evaluating the reactivity of TR proteins with plasma samples from patients with VL, Goto *et al*. (68) also demonstrated that the antibody binding to the antigen is stronger as the copy number of the repeats increases.

This is probably the reason why kinesin-derived proteins are recognized as good antigens for VL diagnosis, since they have a highly immunogenic TR region composed of 39 amino acids, as shown in rK39 (19) and rKDDR (28).The presence of potential B-cell epitopes was also analyzed *in silico* in the new antigen rKDDR-plus. Throughout its sequence the presence of a large number of linear B-cell epitopes coinciding with a long region of structural disorder was observed. These results suggest that the rKDDR-plus antigen is probably capable of interacting with lymphocytes of the host's immune system. These epitopes can be more easily identified during antibody serological screening compared to molecules with fewer repetitions, thus compensating for the low prevalence of antibodies observed in some patients (88).

To evaluate the influence of the number of repetitions from the sequence of the 39 amino acids in kinesin-derived proteins comparative ELISA assays with three proteins were performed. Although the sensitivity of the rK39, rKDDR and rKDDR-plus antigens were the same (98.0% for humans and 96.7% for dogs) in the ELISA, the specificity was proportional to the number of repetitive motifs present in the proteins. Considering that rK39 has 6.5 repeats, rKDDR 8.5 repeats, and rKDDR-plus 15.3 repeats, their specificities were 71.1%, 96.1% and 100.0% for human sera, and 82.5%, 90.8% and 97.5% for canine sera, respectively. Consequently, the antigen with the highest number of repetitive motifs (rKDDR-plus) was also the one that obtained the best accuracy results, with both sera, followed by rKDDR and rK39. Therefore, these results suggest that the increased presence of TR motifs can increase the diagnostic performance of serological tests for VL. The considerable cross-reactivity of the antigens tested with the sera from dogs infected with *Babesia* sp. and *Ehrlichia* sp. could be explained by a possible exposure of these animals to *Leishmania*, since the reference diagnosis used was the parasitological, which may have a lower sensitivity than serological. Even considering this possibility, rKDDR-plus showed an excellent result since this antigen did not show any cross-reactivity with *Ehrlichia* sp. and showed minor reactivity (only 11%) with *Babesia* sp., the lowest value among the tested antigens.

In the ELISA experiments, in addition to the CSA, the rK26 protein was also used as a control. This antigen has 11 repeats of a 14 amino acid sequence that can also be described as two alternating repeats of 7 amino acids (PKEDGH/RT and QKNDGDG) (23). The rK26 has a sensitivity ranging from 21.3% to 96.8% and a specificity from 80% to 100% described in other studies (26, 32, 34, 89). Although the rK26 antigen also has an expressive number of repetitive motifs (11 repeats), greater than the number of repetitions of rK39 and rKDDR, it showed lower values of sensitivity and specificity for human sera (82.0% and 80.3%, respectively) and for canine sera (91.7% and 81.7% respectively), when compared to all antigens derived from kinesin, except for the specificity of rK39 that was lower with human sera.

However, the 14 amino acid repetitive region of rK26 shares no homology with the 39 amino acid repetitive region of proteins derived from kinesin. Therefore, these results suggest that the diagnostic performance of a test depends on both the composition of the TR sequence and its number of copies.

Another advantage attributed to recombinant proteins is their adsorption capacity on various surfaces that can be used in the field. In the last decade, ICT has been used as diagnosis of VL in several countries (17, 90). This test combines the easy execution and rapid interpretation of the results, as well as the independence of laboratory infrastructure and specialized professionals for the execution (21). Therefore, in this study, an ICT based on the rKDDR-plus protein, to be used in the rapid diagnosis of HVL was developed and evaluated. The sensitivity and specificity of the ICT/rKDDR-plus were 89.7% and 100.0%, respectively. The performance of ICT/rKDDR-plus was compared with the commercially available ICT IT LEISH, which is based on the rK39 antigen. Analyzes show that ICT IT LEISH obtained a sensitivity of 79.3% and a specificity of 97.5%. These results show that ICT/rKDDR-plus has a greater ability to detect the disease when it is present and to discard it when it is absent compared to commercial test. The sensitivity of ICT IT LEISH can vary from 81.1% to 96.2%, whereas its specificity varies between 96.6% to 98.7% (20, 91-93). Therefore, it is possible to conclude that the rapid test ICT/rKDDR-plus has great potential to be used as a diagnosis for HVL in field screening, since it presented a better diagnostic performance.

However, the results presented in this study on the use of rKDDR-plus in the VL diagnosis refer to an initial and relatively small-scale trial. In addition, the results obtained for NPV and PPV in the ELISA and ICT tests may differ depending on the prevalence of VL in the endemic areas being tested or sample set with different VL positivity rates. Therefore, tests with a greater number of samples carried out in the field are in progress and will allow us to verify points that could be improved in these diagnostic tests. Currently, the KDDR-plus antigen has been licensed by a company for the commercialization of ELISA and ICT for the diagnosis of human VL, and is in the process of transferring technology for veterinary use.

2.7. Conclusion

As stipulated at the beginning of the chapter, kinesins are an important superfamily of proteins that for over 30 years have revolutionized the diagnosis of visceral leishmaniasis. In addition, as postulated at the end of the chapter, we show that different compositions of amino acid sequences present in this superfamily can influence the diagnostic performance of the disease. We also show that increasing repetitive amino acid sequences in a protein improves the diagnostic performance of the resulting serological tests satisfactorily by increasing its specificity. This discovery may be useful not only for the diagnosis of VL, but it may be beneficial for the discovery of serological markers for other diseases caused by other trypanosomatids, such as Chagas disease. Despite the results obtained in this chapter, resulting in the discovery of a promising antigen, which has its performance improved by increasing its repetitive portions, our efforts to contribute to the control of VL have not ceased. Therefore, the results obtained in this study show that the recombinant antigen

rKDDR-plus is a highly promising candidate for VL diagnosis in both serological tests (ELISA and ICT), presenting the highest accuracy and specificity compared with others kinesin-derived antigens, which are currently used in the diagnosis of this disease. The best diagnostic performance of rKDDR-plus in serological tests was probably due to the increased presence of repetitive motifs in the protein.

2.8. Transition

In this chapter, the new KDDR-plus antigen showed the best specificity values in human and canine ELISA tests and in human ICT. With regard to the sensitivity of these tests, KDDR-plus showed values equal to or greater than the tested antigens. Regarding the canine diagnosis exclusively, when the specificity was analyzed, it is observed that the KDDR-plus presented the best result, with a predictive value substantially higher than the other antigens. Regarding sensitivity, the result was similar between the analyzed antigens. It is known that one of the main factors that interfere with the loss of sensitivity of these antigens are asymptomatic dogs. The asymptomatic form of the disease is a major concern in the diagnosis of CanL and in the transmission control of *Leishmania* infection. When stratifying the group of dogs with leishmaniasis into asymptomatic and symptomatic, based on the presence or absence of clinical signs, it was observed that exclusively dogs without clinical signs of the disease affected the sensitivity of KDDR-plus. Based on this observation, the search for new antigens capable of identifying mostly asymptomatic dogs is still needed because a protein capable of identifying all cases of symptomatic dogs was presented in this chapter. Therefore, the following chapter of this thesis focuses essentially on the group of asymptomatic dogs. Chapter 3 highlights the relevance of dogs without clinical signs of leishmaniasis for the spread of the disease and emphasizes the importance of correctly distinguishing this problematic group of animals from non-infected dogs, mainly in canine population surveys of control programs of the human disease. Thus, there is a need to continue the search for a protein capable of identifying VL in dogs not showing any clinical signs of infection. In the next chapter, we, therefore, investigate a recombinant protein of the dynamin-1-like protein superfamily as a potential candidate to create such proteins.

3. Chapter III - Dyn-1 protein

Serodiagnosis of leishmaniasis in asymptomatic and symptomatic dogs by use of the recombinant dynamin-1-like protein from *Leishmania infantum*: A preliminary study.

Adapted from:

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3.1. Chapter context

One of the main challenges for the control of human and, consequently, canine VL is the identification of dogs infected by *Leishmania*, mainly dogs without clinical signs of the disease. Therefore, this chapter introduces important definitions such as the criteria for characterizing a dog as symptomatic or asymptomatic. Furthermore, another protein superfamily (dynamin) was studied, that until then had not been mentioned as a potential target for the diagnosis of canine leishmaniasis. The sequence of rDyn-1 protein, derived from the dynamin superfamily, was studied using bioinformatics resources. Its diagnostic performance was tested through serological assays against a sera bank containing samples from dogs previously characterized as symptomatic, asymptomatic and others. Finally, their performance was compared with market reference antigens that are widely used in commercial tests. Thus, the results in this chapter will introduce another protein family that offers highly promising VL diagnostic markers.

3.2. Chapter abstract

Visceral leishmaniasis (VL) is a fatal manifestation of an infection caused by intracellular protozoa of the *Leishmania* genus. In New World countries, VL is classified as a zoonotic disease with domestic dogs acting as its main reservoir. Asymptomatic dogs are as competent to transmit *Leishmania* to the vectors as symptomatic dogs; however, current diagnostic tests are limited and present low sensitivity for this important group. The development of accurate tests is fundamental to the early diagnosis, treatment, and control of canine leishmaniasis. In this study, the use of a recombinant protein (dynamin-1-like protein, Dyn-1) from *L. infantum* was investigated as a potential target antigen for leishmaniasis serodiagnosis in both symptomatic and asymptomatic dogs. The antigenic performance of the protein was evaluated by means of ELISA assays using sera from symptomatic (n=25), asymptomatic (n=34) and non-infected dogs (n=36) using ELISA. In addition, sera from dogs infected with *Trypanosoma cruzi* (n=49) and infected with *Babesia* sp. (n=8) were tested to evaluate possible cross-reactivity. A crude soluble antigen (CSA) of *Leishmania* was used as an antigen control and K39 and K26 were used as reference antigens because they are already widely used in commercial tests. The rDyn-1-based assay showed the highest sensitivity (97%) compared to the antigens K39 (88%), K26 (86%) and crude extract (95%). The highest specificity among the tests was also obtained with the protein rDyn-1 (94%), compared with the other antigens K39 (81%), K26 (87%), and crude extract (77%). This study showed that the rDyn-1 ELISA assay was able to identify 100% of asymptomatic dogs, establishing its potential as a target for the diagnosis of canine leishmaniasis.

3.3. Introduction

Visceral leishmaniasis (VL) still holds an endemic status in approximately 80 countries worldwide, remaining one of the highest mortality rate parasitic diseases (94). Transmission occurs almost exclusively by sand fly bite, particularly in areas where the precariousness of housing and basic sanitation favors vector reproduction and the maintenance of the infection cycle (95). This complex cycle can vary according to the geographic region, species of *Leishmania*, and vertebrate and invertebrate hosts involved (96). In Old World countries where VL is caused by *Leishmania donovani*, the disease is classified as an anthroponosis since it occurs mostly in the absence of reservoirs. On the other hand, in the New World, leishmaniasis is considered a zoonosis caused by *L. infantum* species, which inherently establishes the importance of reservoirs in the maintenance and transmission of the disease (97).

Among wild and urban animals, dogs (*Canis familiaris*) are considered the main reservoir of leishmaniasis due to their close relationship with humans (98) combined with the fact that most infections remain asymptomatic and therefore are not timely treated (99). Similar to it occurs with humans, clinical diagnosis of canine leishmaniasis is very difficult as there is often a lack of pathognomonic clinical signs (100-102). In addition, not every infected animal develops clinical manifestations (103). In this context, stand out mainly dogs residing or staying for a long time in areas considered endemic for leishmaniasis, without clinical signs compatible with the disease and that present positive diagnostic in a combination of tests (serological, molecular, and/or parasitological) characterized as asymptomatics (104). Dogs with asymptomatic infections are just as important as dogs with overt clinical manifestations for many reasons. Despite low levels of circulating parasites often observed in asymptomatic dogs they are as capable of transmitting the parasite to the vector with a high rate of parasitic infection as well as symptomatic dogs (105). Thus, these animals play an important role in maintaining the cycle and dissemination of the disease, mainly in endemic areas, presenting a risk to public health (106). Concerning the diagnosis, even though asymptomatic dogs are positive for *Leishmania*, current sorological tests present limitations to identify these animals (107, 108). Therefore, a sensitive and specific test that successfully detects both symptomatic and asymptomatic infections is highly desirable.

Several serological tests are commonly used for the diagnosis of leishmaniasis and also are valuable tools in epidemiological studies, disease control programs, field studies, and laboratories (109). The enzyme-linked immunosorbent assay (ELISA) (110), the latex agglutination test (LAT), the direct agglutination test (DAT) (111), and Immunochromatographic tests (ICT) (112) are some tools currently used for this purpose. DAT has been used extensively over the last decade for the serological diagnosis of leishmaniasis as well as in seroepidemiological studies particularly in Iran and some African countries (113, 114). Attributes such as simplicity and safety, good specificity and sensitivity, economy, and field applicability have led to extensive application of DAT in leishmaniasis endemic regions of Iran (115, 116). DAT is a semi-quantitative assay based on the agglutination of biological material when in contact with the dead *Leishmania* sp. parasite stained (117). However, as

with other serological tests, the DAT has limitations that must be considered (118). Unlike DAT, ELISA has as its principle the coating of plates with antigens of interest that, when in contact with positive samples, are quantified using immunoglobulins labeled with enzymes (117). ELISA and ICT that use crude soluble antigens (CSA) (119, 120) or recombinant proteins from *Leishmania* (2, 76) are commonly employed for leishmaniasis diagnosis (121, 122). Among the antigens explored, kinesin-derived proteins such as rK39 (19), rKDDR (28) and rKDDR-plus (123), are excellent targets with the ability to discriminate both human and canine VL from healthy individuals (19, 28, 123). However, the diagnostic performance of assays targeting kinesin-derived proteins may vary according to the geographical region surveyed and, for canine leishmaniasis, according to the clinical sings (65, 124).

In the search for new recombinant molecules capable of improving the accuracy of serological tests, previous studies performed in this laboratory have demonstrated that housekeeping genes of *Leishmania* have a high potential for the serodiagnosis of leishmaniasis (125, 126). In this study, the performance of a new antigen rDyn-1 was evaluate and compared with crude soluble antigens (CSA) from the culture of *L. infantum* promastigotes, rK39, and rK26 proteins, which are widely used in diagnostic kits. The results obtained in this study demonstrate that ELISA test based on rDyn-1 identified all serum samples from asymptomatic dogs identifying animals in early stages with high sensitivity rates. Additionally, when compared to the other antigens evaluated, rDyn-1 showed less cross-reactivity in ELISA using sera from animals infected with other relevant parasites, such as *T. cruzi* and *Babesia* sp. Taken together, these results demonstrate that rDyn-1 is a highly interesting antigen for CanL immunodiagnostic, especially in asymptomatic dogs.

3.4. Material and Methods

3.4.1. Ethics statement and study design and population

The use of the samples in this study was approved by the Institutional Animal Care and Ethics Committee on Animal Use (CEUA) from the Federal University of Minas Gerais (protocol number 44/2012).

The sera panel consisted of 59 samples from dogs infected with *L. infantum* (25 symptomatic and 34 asymptomatic) obtained from the endemic area for CanL in Montes Claros, Minas Gerais State, Brazil. To confirm the positivity of the animals, samples of bone marrow (BM) and whole blood from all animals were collected. Part of the bone marrow material collected was cultivated in Novy-Mac Neal-Nicolle (NNN) medium supplemented with 20% FBS (Fetal Bovine Serum, Gibco BRL, New York, USA) and 100 µg/mL of gentamicin. The cultures were maintained at 24°C for four weeks and examined weekly for the presence of parasites (127). The remainder of the BM together with the whole blood samples were subjected to DNA extraction techniques followed by specific qPCR assays for *Leishmania* kDNA. The qPCR method for evaluating the parasitic load (DNA copies/µL) in the samples was performed using the qPCR method (Real-Time PCR with TaqMan Probe) based on a probe with a standard curve and direction of the genome kDNA minicircle sequence from *L. infantum* (TECSA Laboratories® , Belo Horizonte, Minas Gerais, Brazil). The absence of clinical signs suggestive of *Leishmania* infection was used to define dogs

with asymptomatic clinical status. On the other hand, dogs that presented some of the most common signs of the disease such as skin changes (alopecia, furfuraceous eczema, ulcers, hyperkeratosis), onychogryphosis, weight loss, keratoconjunctivitis and hindlimb paresis were characterized as symptomatic. It used 36 sera from non-infected dogs (negative samples) from non-endemic areas for the disease confirmed by qPCR assays, specific for *Leishmania* kDNA, from peripheral blood and serological tests (ELISA and ICT) from serum. To evaluate possible cross-reactivity and co-infection cases 49 samples from dogs infected with *T. cruzi* and 8 from dogs infected with *Babesia* sp. were tested. Serum samples infected with *T. cruzi* were kindly provided from the Department of Clinical Analysis of the School of Pharmacy/UFOP. The dogs were inoculated with 2.0×10^3 bloodstream trypomastigotes per kg of body weight belonging to two strains. The Y strain (DTU TcII), isolated from an acute human case of Chagas' disease and the Berenice-78 (Be-78) strain (DTU TcII) isolated by xenodiagnosis of a patient with an indeterminate form of the disease. The positivity to *T. cruzi* was confirmed by hemoculture or by combined positivity indicated by Chagatest-ELISA Recombinante version 3.0 kit (Wiener Laboratorios, Santa Fé, Argentina) and Chagatest Indirect Hemagglutination Assay (IHA; Wiener Laboratorios).

Samples from dogs infected with *Babesia* sp. were kindly provided from a private veterinary laboratory (Contagem/Minas Gerais State, Brazil). The infection with *Babesia* sp. was confirmed by real-time PCR performed after DNA extraction from EDTA-anti-coagulated blood samples in the commercial laboratory. Approximately 6 ml of blood per animal were collected from the saphenous vein of the hind limb or the cephalic vein of the forelimb. The collected blood was distributed into two collection tubes (3.0 ml in each tube), one containing EDTA anticoagulant and the other without anticoagulant for serum separation. Blood stored in EDTA tubes was pipetted and aliquoted into microtubes. The blood contained in the tubes without anticoagulant was centrifuged at 2500 rpm for 10 minutes and the serum resulting from the centrifugation was pipetted and aliquoted into new tubes. Subsequently, all samples were stored at -80ºC.

3.4.2. Selection of the protein and analysis and linear B-cell epitope prediction

A Dynamins are guanosine triphosphatases (GTPase) that are related to several processes linked to membrane dynamics and functioning, having an especially important role in endocytosis (128). The dynamin superfamily in eukaryotic cells includes several molecules involved in numerous intracellular membrane trafficking events, including the Dyn-1 (dynamin-like proteins) (129). The sequence of the dynamin-1-like protein, from *L. infantum* (ID: LINF_290029700), starin JPCM5, used in this work were obtained from the predicted proteomes available in the TriTrypDB database (http://tritrypdb.org), from which a sub-bank was generated after the exclusion of pseudogenes and partial genes. For the selection of possible targets for the serodiagnosis of leishmaniasis, *L. infantum* proteins were chosen based on their similarity with protein sequences related to the host's immune system and/or pathogen defense, deposited on the ImmunoneBase database (130). Similarity analysis was performed using the BLASTp algorithm (131, 132), with an e-value cutoff

of less than 1e-50 was employed for the NCBI (National Center for Biotechnology Information) Reference Sequence (RefSeq) database. Linear B-cell epitopes were predicted using the BepiPred 1.0 program (http://www.cbs.dtu.dk/services/BepiPred-1.0/) (70), with a cutoff of 0.8. Intrinsically unstructured/disordered regions were predicted by IUPred program (http://iupred.elte.hu/) (71) with a cut-off of 0.5.

3.4.3. Total extract of *L. infantum*

A total extract of the parasite was prepared from *L. infantum* (MHOM/BR/1974/PP75) promastigotes maintained at 24 ºC in Schneider's Insect Medium (Sigma-Aldrich, USA), supplemented with 10% inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco/Thermo Fisher Scientific, USA). Approximately 5x108 parasites were washed and resuspended in 1 mL PBS, followed by lysis through 15 cycles of freezing in liquid nitrogen and thawing at 37 ºC. The concentration of the total proteins of the parasite was quantified using the Pierce BCA Protein Assay (Thermo Fisher Scientific, USA).

3.4.4. Cloning, protein expression and purification

The primers used to amplify the entire coding region of the dynamin-1-like protein from *L. infantum* (rDyn-1) were Forward, 5' ACT**CATATG**GACCAGTTGATCAGCGTGATC 3' and Reverse, 5' GTT**GTCGAC**TTAGGCGCCGGCTTGCATGGAC 3'. Restriction enzyme (*Nde*I and *Sal*I, respectively) sites that were added to the sequence to facilitate cloning were shown in bold. The resulting 2.1 kb DNA fragment was excised from an agarose gel, purified, and linked into the pGEM-T Easy vector (Promega, USA). Recombinant plasmid pGEM-rDyn-1 was used to transform *Escherichia coli* XL1-Blue competent cells (Phoneutria, Brazil). Positive clones were confirmed by colony PCR using M13 universal primers, and later for constructing the expression vector. The DNA fragment obtained from the digestion of pGEM-rDyn-1 with *Nde*I and *Sal*I was ligated into pET28a-TEV, previously digested with the same enzymes. The pET28a-TEV expression vector contains a sequence encoding a histidine tag, in their N-terminal region, to facilitate the protein purification process. Electrocompetent *E. coli* ArcticExpress (DE3) cells (Agilent Technologies, USA) were transformed by electroporation using a MicroPulser Electroporation Apparatus (Bio-Rad Laboratories, USA) with the recombinant plasmid pET28a-TEV-rDyn-1. Gene insertion was confirmed by colony PCR and sequencing, using T7 universal primers (Macrogen, South Korea).

Expression of the recombinant rDyn-1 protein was induced in the positive clones by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, USA), for 24 h at 200 rpm and 12 ºC. Cells were ruptured by sonication and centrifuged at 6,000×g for 30 min at 4 °C. The recombinant protein was purified using a HisTrap HP affinity column connected to the ÄKTAprime chromatography system (GE Healthcare, USA).

3.4.5. ELISA serological assay

Through tests, ELISA evaluated the performance of Dyn-1 protein and the other antigens. The rK39 and rK26 antigens were kindly provided by Steven G. Reed (Infectious Disease Research Institute-IDRI, Seattle, Washington). The rDyn-1 recombinant protein, as well as total *L. infantum* extract and the kinesin-derived proteins rK26 and rK39, widely used in commercial diagnostic kits, were coated onto 96-well microplates (Nalge Nunc Intl., USA) overnight at 2-8 ºC, with an amount of 50 ng/well for rDyn-1, 1000 ng/well for total extract, and 100 ng/well for rK39 and rK26. The plates were blocked with 200 µL of 2% PBS-BSA for 1 h at 37 ºC and treated successively with 1:100 dilutions of the canine serum samples for 1 h at 37 ºC. Peroxidase-labeled antibodies specific to dog IgG (Sigma-Aldrich, USA) were diluted at 1:5,000 and added for 1 h at 37 ºC. The wells were washed, and o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, USA) in citrate buffer containing hydrogen peroxide (Sigma-Aldrich, USA) was added. The plates were incubated for 20 min in the dark and reactions were stopped by the addition of 4 N H_2SO_4. The absorbance at 492 nm was determined on an automatic microplate reader (Versamax, Molecular Devices, USA). Each sera sample was assayed in duplicate. The results of the ELISA using rDyn-1 as antigens were compared with total *L. infantum* extract, rK26 and rK39.

3.4.6. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed with GraphPad Prism™ software (version 6.0 for Windows). The cut-off values were calculated by constructing Receiver Operating Characteristic (ROC) curves, which were plotted with the individual OD values of CanL (symptomatic and asymptomatic) dogs group versus those from the other (healthy dogs, with *T. cruzi* and *Babesia* sp.) groups. ROC curves were used to calculate: sensitivity, specificity, area under the curve (AUC), 95% confidence interval (95% CI), positive predictive value (PPV), and negative predictive value (NPV). Through the ROC curve was possible to evaluate all the combinations of sensibility and specificity and determine the best cut-off value based on the most appropriate sensitivity and specificity ratio for each antigen tested. Agreement beyond chance was assessed using the kappa coefficient (95% CI) and interpreted according to the following scale: 0.00 to 0.20 (negligible), 0.21 to 0.40 (weak), 0.41 to 0.60 (moderate), 0.61 to 0.80 (good), and 0.81 to 1.00 (Very good).

3.5. Results

3.5.1. Selection of the *L. infantum* dynamin-1-like protein

For the selection of a potential target of *L. infantum* for use in the serological diagnosis of CanL, proteins of the parasite with protein sequences related to the host's immune system, deposited in the ImmunoneBase database, were analyzed. The

dynamin-1-like protein from *L. infantum* (rDyn-1) was selected for presenting similarities with proteins associated with the host defense processes.

3.5.2. Prediction of linear B-cell epitopes and structural disorder of rDyn-1

To assess *in silico* the antigenic potential of the rDyn-1 protein, the complete amino acid sequence was inserted in the BepiPred and IUPred programs, to predict linear B cell epitopes and structural disorder respectively (Figure 10). The protein showed nine linear B cell epitopes, as well as disordered regions. Since the prediction of B cell epitopes is based on the linear sequence of the protein (primary sequence), the overlap of regions of structural disorder with predicted B cell epitopes increases the possibility that this region of the protein will be recognized by lymphocytes from the immune system of the host.

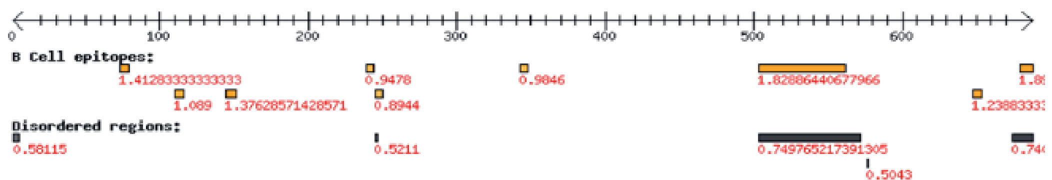

Figure 10 - Predictions of linear B-cell epitopes and structural disorder regions of the dynamin-1-like protein (rDyn-1) from *L. infantum*. The dashed arrow represents the complete amino acid sequence of the rDyn-1 protein (LINF_290029700). The linear epitopes of B cells, predicted by the BepiPred program, were represented by orange boxes. The regions of structural protein disorder, predicted by the IUPred program, were represented by gray boxes. The value below each box corresponds to the score of each prediction.

3.5.3. Expression and purification of the rDyn-1 protein

The full-length coding region of the *L. infantum* rDyn-1 protein was amplified by PCR, cloned into the vector pGEM, and confirmed by sequencing. The gene was then transferred to the pET28a-TEV expression vector, and the rDyn-1 was expressed in *E. coli* BL21 and purified by affinity chromatography. A 78 kDa band referring to the rDyn-1 protein was observed by polyacrylamide gel electrophoresis (SDS-PAGE) of the expression in bacteria and the purified fraction as seen in Figure 11.

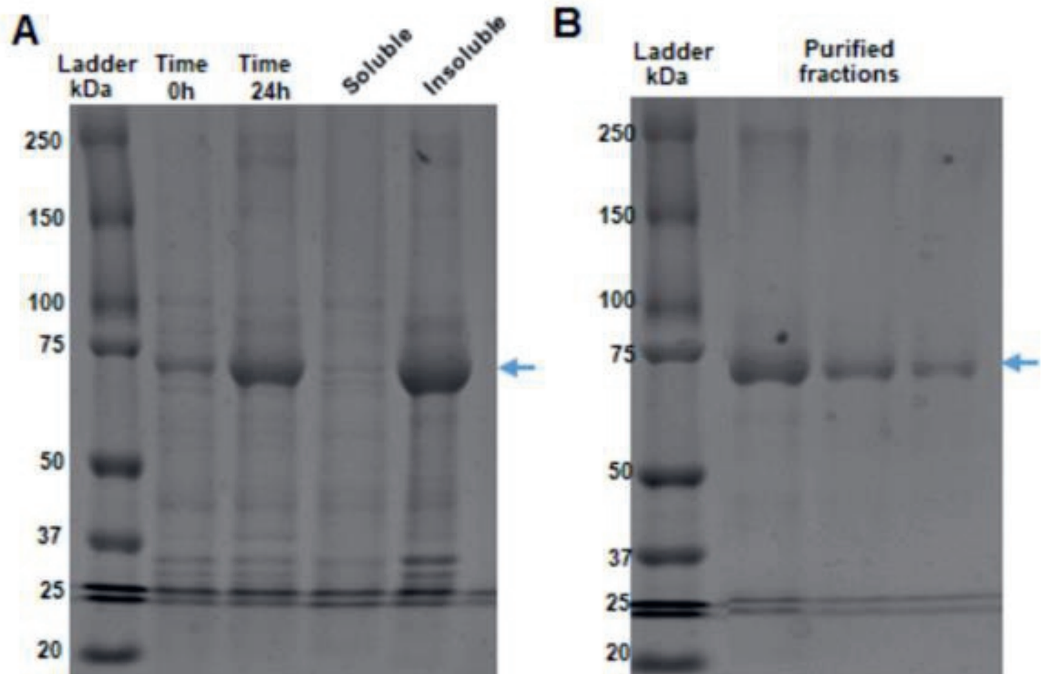

Figure 11 - Analysis in polyacrylamide gel electrophoresis (SDS-PAGE) of the expression in bacteria and purification of rDyn-1 protein. (A) Extracts of *E. coli* BL21 Star containing the plasmid pET28a-TEV/rDyn-1, before (0h) and after (24h) induction of the recombinant protein with IPTG. (B) After purification by affinity chromatography of the insoluble fraction of the bacterial lysate, the purified fraction presented a band of approximately 78 kDa, corresponding to rDyn-1. The blue box indicates the rDyn-1 protein band on the gels. Ladder: molecular mass marker; kDa: kilodalton.

3.5.4. Serological recognition of rDyn-1 by ELISA for the diagnosis of canine leishmaniasis

After purification by affinity chromatography, the recombinant rDyn-1 protein (LINF_290029700) was used as an antigen in an ELISA experiment to assess its reactivity against canine sera. In Figure 12, the rDyn-1 protein showed an improved performance against canine sera in the ELISA experiment, when compared to the antigens K26 and K39 and the crude extract of *L. infantum*. The assay using rDyn-1 as an antigen proved to be the only one capable of detecting 100% of asymptomatic infected dogs for CanL and also showed the lowest percentage of cross-reactivity with dogs infected with *T. cruzi* and *Babesia* sp. The K39, K26 and crude extract antigens identified only 79%, 76% and 94% of asymptomatic dogs, respectively, and showed greater cross-reactivity mainly with dogs infected with *T. cruzi*. The antigens K39 and K26 were also recognized in 6% of healthy dogs (negative control).

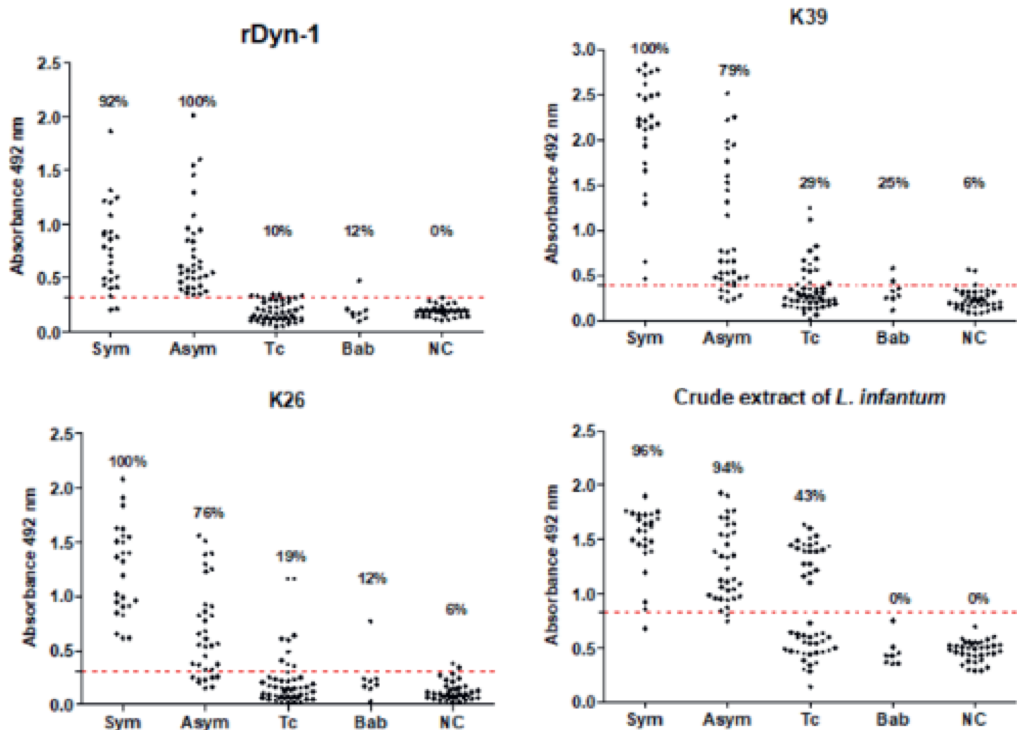

Figure 12 - Evaluation of the recombinant rDyn-1, K39 and K26 antigens, and *L. infantum* crude extract for the serodiagnosis of CanL. Comparison of the performance of ELISA was performed using canine sera from the following groups: dogs infected with *Leishmania*, 25 symptomatic (Sym) and 34 asymptomatic (Asym); 36 non-infected healthy dogs (NC); 49 infected dogs with *T. cruzi* (Tc); and 8 infected dogs with *Babesia* sp. (Bab, n=27), to evaluate possible cross-reactivity. The dashed red line represents the cut-off determined by the ROC curve of each test. The index above each column in the plot indicates the percentage of samples that were above the cut-off.

Figure 13 shows the ROC curve used to determine the cut-off for each antigen and the area under the curve (AUC). The rDyn-1 protein assay showed the highest AUC value (0.9858), followed by K26, rK39, and the crude extract of *L. infantum*, which presented the values 0.9405, 0.9196, and 0.8917, respectively.

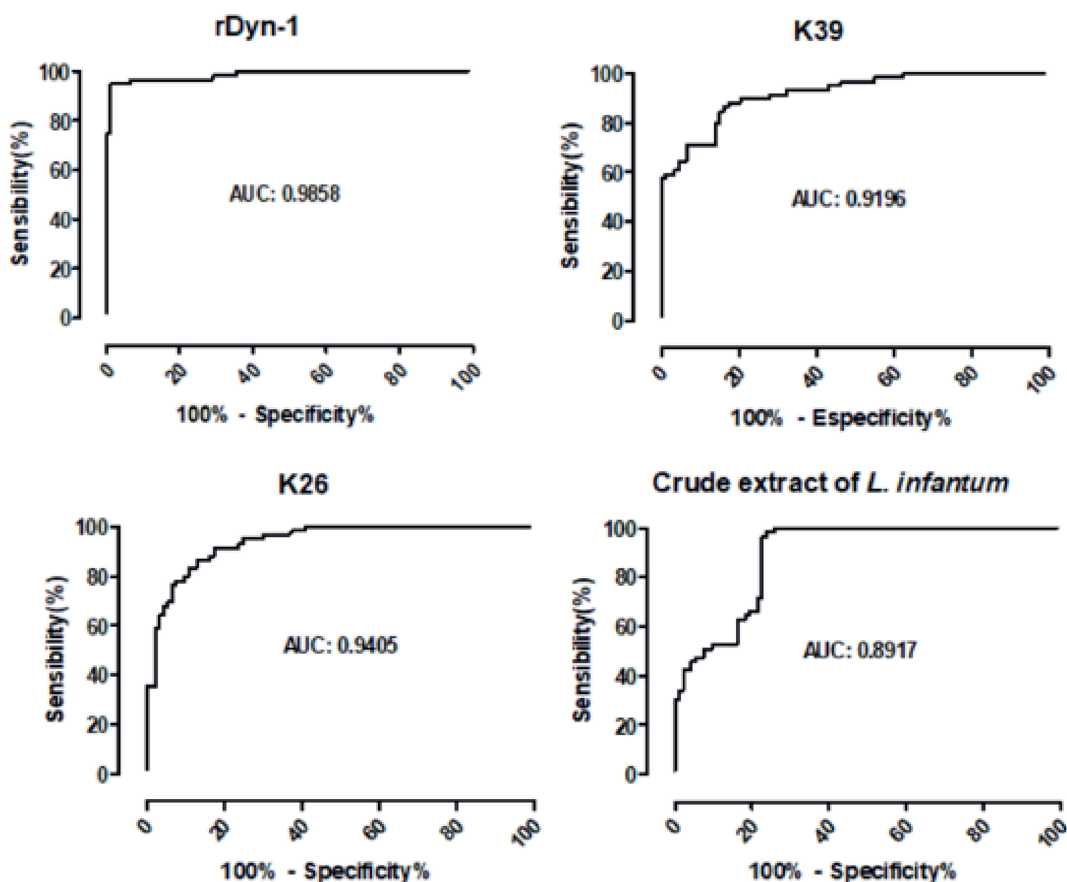

Figure 13 - Comparison of ROC curve of each canine serological test obtained for ELISA using recombinant rDyn-1, K39 and K26 antigens, and crude extract of *L. infantum*. The sensitivity, specificity and area under the curve (AUC) were determined by ROC curve.

When examining the diagnostic performance of the assays, the rDyn-1-based assay showed the highest sensitivity and specificity (97% [IC 95%; 88.3% - 99.6%] and 94% [IC 95%; 86.5 - 97.6%], respectively) compared to the essays using antigens K39 (88% [IC 95%; 77.1 - 95.1%] and 81% [IC 95%; 71.2 - 88.1%], respectively), K26 (86% [IC 95%; 75.0 - 94.0%] and 87% [IC 95%;78.6 - 93,2%], respectively) and crude extract (95% [IC 95%; 85.9 - 98.9%] and 77% [IC 95%; 67.6 - 85.5%], respectively) (Table 6). In addition, accuracy, PPV and NPV values the rDyn-1 antigen were also bigger 95%, 90% and 98%, respectively compared to the other antigens.

Table 6 - Diagnostic performance of ELISA tests, using recombinant rDyn-1, K39 and K26 antigens, and *L. infantum* crude, to detect antibodies against CanL.

ELISA Test	Cut-off[α]	Sensitivity (%) [CI 95%; n= 59]	Specificity (%) [CI 95%; n=93]	PPV (%)	NPV (%)	AC (%)
rDyn-1	0.3228	97 [88.3% - 99.6%]	94 [86.5% - 97.6%]	90	98	95
K39	0.3964	88 [77.1% - 95.1%]	81 [71.2% - 88.1%]	74	91	84
K26	0.3142	86 [75.0% - 94.0%]	87 [78.6% - 93.2%]	81	91	87
Crude extract	0.8208	95 [85.9% - 98.9%]	77 [67.6% - 85.5%]	73	96	84

Abbreviations: (CI) confidence interval; (PPV) positive predictive value; (NPV) negative predictive value; (AC) accuracy.
[α]Cut-off calculated based on ROC curve.

The highest kappa agreement index was also obtained by the rDyn-1 protein (0.891), showing a very good degree of agreement, suggesting its potential use for canine serodiagnosis as shown in Table 7.

Table 7 - Agreement analysis of ELISA tests using rDyn-1, rK39, rk26 and crude extract with the parasitological diagnosis of CanL.

ELISA Test	AUC	tP	FN	tN	FP	K* [CI 95%]	Agreement**
rDyn-1	0.986	57	2	87	6	0.891 [81.7 - 96.4]	Very good
K39	0.920	52	7	75	18	0.665 [54.7 - 78.4	Good
K26	0.941	51	8	81	12	0.726 [61.5 - 83.8]	Good
Crude extract	0.892	56	3	72	21	0.685 [57.3 - 79.7]	Good

Abbreviations: (AUC) area under the curve; (CI) confidence interval; (tP) test positive; (tN) test negative; (FP) false positive; (FN) false negative; (k) Kappa index.
*To calculate Kappa index, all samples of this work were considered (CanL, n = 53; NC, n = 93).
**Parasitological and qPCR diagnostic assay were the gold standard test for calculating the Kappa index.

3.6. Discussion

Dogs with asymptomatic *Leishmania* infections are more present than symptomatic dogs in the population of endemic areas. However, research has shown there was no statistically significant difference in the rate of vector infectivity between symptomatic and asymptomatic dogs (133, 134). In those regions, infected but asymptomatic dogs may not be adequately diagnosed by current serological tests,

due to the low number of antibodies produced by the host's immune system or because it is the initial stage of the disease (135). In Brazil, the governmental policy to control human infection based on the identification of seropositive dogs (130, 136). The Brazilian Ministry of Health officially established a rapid chromatographic immunoassay for canine survey based on dual path platform (DPP®) for screening of the disease and ELISA as a confirmatory test (137). Serologic tests, currently, are methods of choice in population studies because it is a relatively simple and fast tool that can be performed manually or automated (56, 121), and it allows a large number of samples to be analyzed simultaneously in a short period. The key to controlling *Leishmania* infection and associated diseases in the human population, therefore, it's in the development of new, improved serological tests that are able to detect *Leishmania* infection in one of their main reservoirs, dogs that do not display any clear symptoms of infection (11).

The prevalence of CanL is often underestimated, mainly due to the fact that the detection of antibodies in dogs considered asymptomatic or with recent infections is generally lower in serological tests (138). For years, crude extracts have been used in the serological diagnosis of leishmaniasis mainly in ELISA-based assays. Despite the relatively low cost of production of the crude extracts, the results indicate that although ELISA assays using this antigen demonstrated a relatively high degree of sensitivity when used to identify both asymptomatic and symptomatic dogs (95% and 94%, respectively) their major limitation was the high rate of cross-reaction of dog sera with *T. cruzi.* Resulting in sub-optimally low specificity levels (77%). The use of recombinant antigens, such as kinesin-derived proteins in the serological diagnosis of CanL allowed a better standardization of the assays and therefore more robust results. Among them, the rK39 (19) is the most widely used protein from CanL testing. However, literature data indicate that the protein rK39 was more sensitive for the diagnosis of symptomatic cases (100%) compared to asymptomatic (66%) (139). The results obtained in the work confirm that rK39 antigen-based assays have a good capacity to identify dogs with active disease (100%), but their performance drops considerably when detecting asymptomatic infections (79%). In addition, a high rate of cross-reactions with sera from dogs with *T. cruzi* (29%) and *Babesia* sp. (25%) was still observed. The rDyn-1 antigen-based assay developed in this study has been demonstrated to overcome most of these problems, as its sensitivity and specificity were superior to those obtained with assays based on the rK39 antigen. Furthermore, the recombinant K26/HASPb1 protein, also included in this work as a reference technique, was considered an antigen capable of increasing the diagnostic accuracy of leishmaniasis (140). A study conducted in Iran indicated a sensitivity of 96.8% and a specificity of 100% for this antigen (141). Another study conducted in an endemic area in northwestern Brazil showed that the rK26 antigen has high sensitivity in dogs with symptoms, but low sensitivity in asymptomatic dogs, (94% and 64%, respectively) (139). Following the literature data, the rK26 protein in this study showed high sensitivity (100%) in symptomatic dogs and low sensitivity (76%) in dogs without symptoms. Following the low values of sensitivity and specificity recorded in the literature, in this study, overall sensitivity and specificity of 87% and 87.1%, respectively were found.

The efficacy of assays based on the different recombinant antigens already tested in the literature, K26 and K39 (2, 139, 141, 142), the total extract of the parasite of *L. infantum* promastigotes, and the antigen produced in this study (rDyn-1) were compared. Although a bank of sera with a limited number of samples previously characterized from a single area considered endemic for CanL in Brazil was used, not being representative of the universal population the rDyn-1 protein, a new antigenic protein derived from *L. infantum*, was studied here for first time in terms of application in ELISA-based CanL diagnosis. The assay based on the new antigen showed excellent performance, given its high ability to identify asymptomatic dogs (100%), without losing the ability to detect symptomatic dogs (92%). Overall, the ability of this protein to identify dogs with CanL was 97% [IC95%; 88.3 - 99.6%] (sensitivity).

In addition to the problematic diagnosis of asymptomatic dogs (leading to a limited sensitivity), current diagnostic tests also suffer from cross-reactivity. Antibodies against *T. cruzi* have been recognized using conventional serological methods as the main cause of cross-reactivity with *Leishmania* due to the phylogenetic similarity between *Leishmania* sp. and *T. cruzi*, which poses a problem for overlapping endemic areas (143, 144). Researchers have often inferred the existence of cross-reaction in serological assays for detecting canine leishmaniasis in dogs co-infected with *E. canis or B. canis vogeli* (145). In fact, Mancianti *et al*. (146) demonstrated cross-reactivity of *B. canis vogeli* with anti-*Leishmania* antibodies using dot-ELISA. Although rDyn-1 showed cross-reactivity with other diseases, the specificity (94% [IC95%; 86.5 - 97.6%]) of this protein remained good and higher than the other antigens compared (CSA, rK39 and rK26).

3.7. Conclusion

The results in this chapter show that rDyn-1-based ELISA assays could be advantageous over the classic diagnostic test that lacks the sensitivity to properly diagnose asymptomatic carriers, which is a major limitation to epidemiology studies and hence for control programs (147, 148). Thus, a rapid, sensitive, and specific tool for the detection of *L. infantum* infection in dogs would be highly desirable because it would allow for effective control interventions in areas where zoonotic VL is endemic. The data presented here suggest that further studying the antigenic properties of each protein by carefully analyzing the portions/fragments responsible for its strength and subsequently combining the supremum of each protein in a multiple fragment model may be the way to a 100% accurate diagnosis capable of recognizing all *Leishmania* infections (symptomatic and asymptomatic), without cross-reactions with other diseases. Furthermore, the use of rDyn-1 might be considered in combination with other recombinant antigens (e.g. rK26, rK39, rKDDR, among others) that are remarkable to detect parasitism in symptomatic animals, which might provide an improvement of overall sensitivity that is often impaired when detecting infection in asymptomatic dogs.

3.8. Transition

This chapter has confirmed the hypothesis that the dynamin-1-like protein superfamily should be incorporated into studies for developing targets for leishmaniasis diagnosis. Furthermore, the studies in this chapter have also led to the development of an antigen derived from this new family of proteins, capable of recognizing dogs infected by *Leishmania*. The rDyn-1 particularly stands out in its ability to identify VL in asymptomatic dogs. This can be considered to be a major breakthrough as the inability of diagnostic tests to detect VL in asymptomatic dogs has shown to be the major challenge in terms of VL disease control, especially in low- and middle-income settings. However, it should be noted that the rDyn-1 protein was less accurate in the diagnosis of dogs infected with *Leishmania* that did display clinical signs of the disease. Reflecting back on the results presented in chapter 2, where the KDDR-plus was identified as a target that could identify VL in symptomatic dogs precisely, we tried to combine the insights obtained in chapter 2 and 3 in the fourth and final chapter of the thesis. The protein composition of KDDR-plus and rDyn-1 will be studied to identify the peptide sequences responsible for the positive attributes of both targets. This will allow for the development of a multi-peptide antigen that is able to detect VL in both symptomatic and asymptomatic disease carriers.

Chapter IV - Immunogenic epitopes from Dyn-1 and KDDR-plus proteins

Immunogenic mapping of rDyn-1 and rKDDR-plus proteins and selection of oligopeptides by immunoblotting for the diagnosis of *Leishmania infantum*-infected dogs.

Adapted from:

Siqueira WF, Cardoso MS, Fraga VG, Ottino J, Ribeiro VM, Gondim CN, Barçante JMP, Gomes ACA, Galdino AS, Eersels K, Grinsven BV, Bartholomeu DC, Bueno LL, Cleij T, Fujiwara RT. Immunogenic mapping of rDyn-1 and rKDDR-plus proteins and selection of oligopeptides by immunoblotting for the diagnosis *of Leishmania infantum*-infected dogs. PLoS Neglected Tropical Diseases, v. 17, p. e0011535, 2023.

4.1. Chapter context

In this chapter, a more in-depth study was carried out on the amino acid sequences of the Dyn-1 and KDDR-plus proteins, in order to identify the peptide portions responsible for the good performance of both protein markers in the diagnosis of VL. The results summarized in Chapter 3 of this work showed that Dyn-1 has the ability to identify all cases of dogs infected with *Leishmania* with asymptomatic clinical status. The KDDR-plus protein, as presented in Chapter 2, presents less cross-reaction rates, thus increasing the specificity of VL diagnosis. The use of peptide moieties, derived from proteins that have been shown to be potential diagnostic markers, seems promising. Peptides are smaller molecules and have several benefits compared to larger antigens. They are characterized by their good stability, the absence of sequences indifferent to their performance, their low complexity, and the potential low cost scaling of their production process. On the other hand, individual peptide sequences may be less immunogenic. Therefore, in this chapter, in addition to peptides alone, a mixture of peptides was also evaluated as a marker. At the end of the chapter, a more specific study was suggested with different peptide combinations or the construction of a chimeric antigen combining the supremum of each previously identified and evaluated peptide.

4.2. Chapter abstract

Endemic in Brazil, visceral leishmaniasis (VL) is a zoonotic infection that is among the most important parasitic diseases transmitted by vectors. Dogs are the main reservoirs of canine leishmaniasis (CanL) and their identification is used in some countries as part of disease prevention and control measures in the canine and human population. In this context, serological tests are necessary, composed of antigens capable of correctly identifying infected dogs, minimizing the number of false-negative cases. This study aimed to identify more immunoreactive peptides derived from two previously described whole proteins (rDyn-1 and rKDDR-plus) and compare their performance to the control antigens rK39 and the crude soluble antigen (CSA) for the detection of dogs infected with *L. infantum*, especially the asymptomatic ones. The three selected peptides and a mixture of them, along with the rDyn-1, rKDDR-plus, rK39, and CSA antigens were evaluated using indirect ELISA with sera samples from 186 dogs with CanL, being asymptomatic (n = 50), symptomatic (n = 50), co-infected (n = 19), infected with *Babesia* sp. (n = 7), *Ehrlichia* sp. (n = 6), *T. cruzi* (n = 20) and uninfected (n = 34). The results showed that the rDyn-1 protein and the peptide mixture had the highest sensitivity (100% and 98.3%, respectively) and specificity (97.0 and 98.5, respectively). A high degree of kappa agreement was found for rDyn-1 protein (0.977), mixed peptides (0.965), rKDDR-plus protein (0.953), K-plus peptide 1 (0.930) and Dyn-1 peptide (0.893). The mixture of peptides showed the highest likelihood (65.9). The ELISA using the mixture of peptides and the rDyn-1 protein showed high performance for CanL serodiagnosis. More mix combinations of the peptides and additional extended field tests with a larger sample size are recommended.

4.3. Introduction

Visceral leishmaniasis (VL), also known as Kala-azar, is a serious disease with a wide spectrum of clinical manifestations, which can lead to the death of the individual if not diagnosed and treated early. The VL is on the list of neglected tropical diseases and is considered a disease of high impact on public health, affecting thousands of dogs and humans annually (149). This disease is responsible for about 50,000 to 90,000 new cases annually, of which only 25 to 45% are reported to the WHO (World Health Organization) (150). Ten countries are responsible for more than 90% of VL cases in the world, including Brazil, China, Ethiopia and India (151).

In the Americas, VL is a zoonosis caused by the obligate intracellular protozoan parasite *L. infantum*, and domestic dogs (*Canis familiaris*) are considered the main source of infection (152). The *L. infantum* infection in dogs named canine leishmaniasis (CanL) is a multi-systemic disease with a range of non-specific clinical signs that when present can vary from lymphadenomegaly, splenomegaly, cutaneous and ocular lesions to nephropathies (143, 153, 154). There is a direct association between the number of cases of human and canine disease because, despite the data indicating a higher prevalence of the disease in dogs than in humans, the presence of infected animals contributes to the increased risk of human disease (155). Therefore, the appearance of cases of VL in humans is preceded by canine enzootic.

Animals with CanL are mostly asymptomatic, about 80%, and may not be correctly diagnosed by serological tests because they have a low, fluctuating, or even absent antibody response to the parasite antigens or because they are in the initial phase of the disease (156, 157). Asymptomatic dogs or with unapparent infections are those in which there is no evidence of clinical manifestations or absence of clinical signs suggestive of *Leishmania* infection residing or staying for a long time in areas considered endemic for leishmaniasis, and that present positive diagnostic in a combination of tests (serological, molecular, and/or parasitological) (104). Although the clinical disease is unapparent in these animals, they are bearers of *Leishmania*, being, therefore, an important source of disease reservoirs, being as capable of transmitting the parasite to the vector as well as the symptomatic dogs. (105, 133, 158). Therefore, tests capable of diagnosing early infections in dogs with leishmaniasis, especially asymptomatic dogs, has a significant role in the management and control of human and canine disease.

Parasitological methods, based on direct microscopic visualization or culture of the parasite, are considered the gold standard for diagnosing the disease, as they have 100% specificity (54). However, their sensitivity is highly variable, they are dependent on biological material from aspiration biopsy of the spleen, bone marrow or lymph nodes, making them highly invasive and risky, in addition to not having applicability in the field (1). In turn, serological methods appear as an alternative to invasive parasitological methods, since VL is characterized by a large production of specific antibodies (54). A vast number of serological techniques are available for the diagnosis of the disease, allowing a wide spectrum of sensitivity and specificity, excellent cost-effectiveness and applicability in the field and in mass (159). Indirect Fluorescent Antibody Test (IFAT), Enzyme Linked Immunosorbent Assay (ELISA), Direct Agglutination Test (DAT), Immunochromatographic (ICT) strip are serological

tests most used in the diagnosis of the disease. ELISA and ICT are simple, accurate and efficient serological tests whose performance is mainly related to the type of antigen used. The use of CSA (crude soluble antigen) in this technique has been shown to be a limiting factor of its specificity, in this context, recombinant proteins appear as an alternative. In addition to improving diagnostic accuracy, recombinant proteins allow large-scale production in a standardized way and independent of *Leishmania* growth in laboratory cultures (2).

In this fourth chapter, the complete amino acid sequences of the proteins evaluated in chapters 2 and 3 have been thoroughly studied in order to identify small peptide fragments responsible for the advantages observed in each protein. In Chapters 2 and 3 it has been demonstrated the potential of two recombinant antigens (Dyn-1 and rKDDR-plus), derived from *L. infantum* for the diagnosis of CanL belonging to two protein superfamilies dynamin-1-like and kinesin, respectively. The protein rDyn-1 showed an excellent ability to identify asymptomatic dogs (160). While that, the protein rKDDR-plus presented excellent for discriminating dogs that have the infection regardless of the clinical status of the animal, presenting low cross-reaction with other organisms (123). Both the dynamin-1-like and kinesin protein superfamily are involved in several cellular processes essential for cells reflecting in dynamic properties, similar to molecular motors (26, 161). Besides the rKDDR-plus, other Kinesin-related conserved recombinant antigens have already been reported in the literature as potential targets for the diagnosis of CanL. In this scenario, stand out the antigens rK39 (19), rKDDR (28), rK9 and rK26 (23), rK28 (26), rKRP42 (25), rKE16 (24) and rKLO8 (20). Already antigens derived from dynamins were linked to the diagnosis of CanL for the first time in Chapter 3.

In view of the promising results obtained by both proteins, the peptides were identified, which are present in the rDyn-1 protein, responsible for the ability to recognize the asymptomatic dogs, as well as the symptomatic ones. In addition, the peptides were identified, which are present in the rKDDR-plus protein and are responsible for the ability to discriminate dogs that have *L. infantum* infection maintaining a low cross-reactivity index with other organisms. Therefore this study aimed to identify peptide sequences present in two complete proteins, rDyn-1, and rKDDR-plus, and to select potential immunoreactive candidates to sera from dogs with *Leishmania* to be used in the serodiagnosis of dogs with CanL, mainly in asymptomatic dogs.

4.4. Methods

4.4.1. Ethics statement

For the selection of immunoreactive peptides in the immunoblotting assays, previously characterized canine sera were used. Exclusively a veterinarian, in accordance with article 3 of Resolution No. 877 of February 15, 2008, of the CFMV, performed all collection procedures and anesthetic procedures. The clinical data and samples from dogs with CanL, as well as the permission for data use, were provided by the Ethics Committee on Animal Use of the Federal University of Minas Gerais, Brazil (CEUA) under protocol number 44/2012.

4.4.2. Serum samples

For this study, 186 serum samples from male and female dogs of various breeds and ages belonging to a shelter or not were used. Veterinarians with the consent of the owners or shelter representatives obtained biological samples and performed a physical examination of each animal. Dogs with positive molecular and serological tests for *L. infantum* were included in the group of dogs with CanL; this group in turn was subdivided into two other groups. Dogs with any suggestive sign of the disease such as skin changes (alopecia, furfuraceous eczema, ulcers, and hyperkeratosis), onychogryphosis, weight loss, keratoconjunctivitis, and hindlimb paresis were characterized as symptomatic. On the other hand, the absence of clinical signs suggestive of *Leishmania* infection was used to define dogs with asymptomatic clinical status.

Sera from 100 dogs (50 symptomatic and 50 asymptomatic), infected from endemic areas of Brazil (Montes Claros, Minas Gerais State, Brazil) to CanL and with the presence of the disease vector were included. In addition, 19 dogs co-infected with *Babesia* sp. and *L. infantum* with positive serological and molecular diagnoses were included. These dogs had a positive parasitological exam in the microscopic analysis of bone marrow aspirates Giemsa stained, by qPCR assays specific for *Leishmania* kDNA and seropositive for anti-*Leishmania* antibodies by means of ELISA and rapid tests.

Samples from 33 seronegative dogs for anti-*Leishmania* antibodies (ELISA and rapid tests), but infected and with a positive diagnosis for other agents or affected by other confirmed pathological conditions were used to evaluate possible cross-reactions. This group was composed of infected with *Babesia* sp. (n=7) and *Ehrlichia* sp. (n=6) and dogs infected with *T. cruzi* (n=20). Samples from dogs infected with *Babesia* sp. and *Ehrlichia* sp. were kindly provided from a private veterinary laboratory (Contagem/Minas Gerais State, Brazil). The infection was confirmed by real-time PCR performed after DNA extraction. Serum samples infected with *T. cruzi* were kindly provided by the Department of Clinical Analysis of the School of Pharmacy/UFOP. The dogs were inoculated with 2.0×10^3 bloodstream trypomastigotes per kg of body weight belonging to two strains; Y strain (DTU TcII), isolated from an acute human case of Chagas' disease, and the Berenice-78 (Be-78) strain (DTU TcII) isolated by xenodiagnosis of a patient with an indeterminate form of the disease. The positivity to *T. cruzi* was confirmed by hemoculture or by combined positivity indicated by the Chagatest-ELISA Recombinante version 3.0 kit (Wiener Laboratorios, Santa Fé, Argentina) and Chagatest Indirect Hemagglutination Assay (IHA; Wiener Laboratorios).

Sera from 34 clinically healthy dogs seronegative by the tests recommended by the Brazilian Ministry of Health (ELISA assays and DPP) from areas where CanL is regarded as non-endemic was also included.

4.4.3. Selection of the peptides derived from rDyn-1 and rKDDR-plus from spot synthesis

A bioinformatics prediction along the complete amino acid sequence of the rDyn-1 protein to identify the potentially immunoreactive peptide sequences was

performed. rDyn-1 was subjected to B cell epitope predictions using the BepiPred 2.0 program (http://www.cbs.dtu.dk/services/BepiPred-2.0) with a cut-off of 0.6 (70). Protein structural disorder prediction was performed using the IUPred program (http://iupred.elte.hu/), with a cut-off of 0.5 (71). For the selection of peptides derived from the rKDDR-plus protein, other analyzes were performed.

The rKDDR-plus protein is characterized by the presence of 15.3 blocks of a sequence of 39 amino acids that are mostly repeated. However, these blocks present some degenerations, always varying between two amino acids in some sites. Therefore, in order to choose the peptides that were synthesized in the membrane, a vertical alignment of the 15.3 repetitive blocks was performed, the degenerate sites were identified and the amino acids that were most repeated in these sites were defined to compose the template sequence of 39 amino acids for the following analyses. Then, the 39 amino acids of the template sequence were fractionated into 10 amino acids with a sliding window of 2 amino acids and thus a simple mapping of the complete protein block for the choice of epitopes was performed. Both the peptides derived from the rDyn-1 protein, identified by bioinformatics predictions and the peptides derived from the rKDDR-plus protein, obtained in the scan, were subsequently synthesized in cellulose membranes.

4.4.4. Spot synthesis in cellulose membrane

The peptide sequences, derived from the complete rDyn-1 and rKDDR-plus proteins were synthesized in a cellulose membrane, using an automatic synthesizer ResPep SL (Intavis) and the program MultiPep (Intavis), according to the SPOT synthesis technique (162, 163). To this end, pre-activated and derivatized cellulose membranes were used for the multiple synthesis of peptide sequences in each spot in an automatic peptide synthesizer (Abimed Spot Synthesis–ASP222, Langenfeld, Germany) following the amino acid distribution plan, as well as the determination of the protocols of the various peptides defined in a computer program. Free hydroxyl groups on the cellulose membrane serve as an anchor point for peptide synthesis. These groups were coupled through a stable connection with 8 to 10 ethylene glycol units (Intavis AG), with the aim of moving the peptide away from the support and providing better stability in the connection of the peptide to the membrane. Peptide synthesis always started at the C-terminus of the last amino acid of the established sequence. Free hydroxyl groups on the cellulose membrane served as an anchor point for peptide synthesis. The amino acids were then activated by DIC (diisopropylcarbodiimide) Oxyma Pure at 1.1 M and deposited on the membrane in two coupling cycles without a new deprotection step between them. Free or unreacted NH_2 functions were acetylated (3% acetic anhydride in DMF) in order to avoid side reactions with subsequently added amino acids or other undesirable bonds. The newly coupled Fmoc protecting group of the amino acid was again eliminated in the basic medium by piperidine at 25% v/v 4-methylpiperidine in DMF. A new amino acid dispensing cycle was then performed. These steps were repeated until the last amino acid was incorporated into the peptide being synthesized. At the end of the synthesis, the amino acid side groups were deprotected by the addition of 95% (v/v) trifluoroacetic acid (TFA) associated with 2.5% v/v water and 2.5% (v/v)

triisopropylsilane (TIPS) to remove the protecting groups from the amino acid side chains, and thus the peptides remain covalently attached to the membrane. Afterwards, the membrane was washed 4 times with dichloromethane (DCM), 4 times with DMF, and 2 times with ethanol. The membrane was dried, and the stains were checked under ultraviolet light. The membrane with the various identified peptides was subsequently analyzed by immunoblotting immunoassays using pools of canine sera.

4.4.5. Screening of SPOT membranes

An immunological screening, by means of immunoblotting assays, was carried out to evaluate the serological performance of the peptides synthesized in the cellulose membrane. Initially, the membranes were incubated with pools of sera considered controls, with the first incubation performed with the pool of sera from dogs not infected with *L. infantum* (healthy control), followed by the pool of sera from dogs infected with other parasites; as *T. cruzi*, then with the pool of *Babesia* sp., and finally the pool of dogs infected with *Ehrlichia* sp. After incubations with pools of control sera, incubations were performed with pools positive for *L. infantum*, first with the asymptomatic group and lastly with the symptomatic group. Each pool was used in immunoblotting assays separately and each membrane was incubated with the respective pools individually and on different days. For this purpose, the membrane was blocked with a PBS solution containing 5% BSA and 4% sucrose for 12-16 h under agitation. Then, it was washed 3 times with the washing solution (PBS + 0.1% Tween20) for 10 minutes and incubated for two hours with a pool of specific serum diluted in the proportion 1:500 in the washing solution (PBS + 0.1% Tween20). After incubation, 3 more 10-minute washes were performed with the washing solution, followed by incubation for 1 h with peroxidase-conjugated anti-dog IgG secondary antibody (Sigma-Aldrich), diluted 1:10000. The membrane was again washed 3 times with the washing solution and finally revealed by chemiluminescence, through the addition of Luminata Forte Western HRP substrate (Merck), using the ImageQuant LAS 4000 digital imaging system (GE Healthcare). After development, the membranes were regenerated (removal of all antibodies bound to it) so that it could be used again with another pool of sera. For this, the membranes were initially washed 3 times using NN'Dimethylformamide (DMF) for 10 minutes, followed by incubation for 12 to 16 hours with a denaturing solution (8M urea + 1% (v/v) SDS in milli-Q water) and more two washes with the denaturing solution for 30 minutes each, followed by three washes with an acid solution (55% (v/v) ethanol, 35% (v/v) milli-Q water, 10% (v/v) glacial acetic acid). Finally, the membrane was washed for 2 minutes with deionized water, followed by 2 washes of 5 minutes with ethanol to remove moisture, dried at room temperature, and stored dry at 4°C or promptly reused. It is also worth mentioning that each membrane can be reused about 30 to 40 times with different sera, allowing the identification of reactive peptides (162).

4.4.6. Scanning and measurement of spot signal intensities

Densitometric analyzes were performed for a better visual comparison, restoring the staining pattern of the spots, normalizing the colors for the stain with the highest value in all membranes, and allowing a real comparison between them. For that, ImageJ software and the Protein Array Analyzer plugin (http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ.html) were used. The densitometry values of each stain were calculated using color intensity. Thus, it was possible to characterize the reactivity of the peptides by comparing the five types of sera tested. In this case, as the color progresses on the scale, the greater the reactivity of that peptide. After densitometry analysis, the next step was to start the process of selecting peptides based on their specificity and reactivity. For this, some filtering and selection criteria were assigned to obtain potential targets. Criterion 1: the background color of the membrane was removed, that is, the four least reactive spots were selected, and the average was calculated followed by the subtraction of this value in each spot by this value. Criterion 2: exclusion of peptides that were reactive in the incubation with the pool of negative control sera, that is, peptides above the established cut-off (negative mean + 2x the standard deviation). Criterion 3: exclusion of poorly reactive peptides in the incubation with the pool of sera with *Leishmania*, that is, peptides with positive spots smaller than the cut-off (mean of the negatives + 3x the standard deviation). These selection criteria allowed an initial screening to eliminate the peptides of lesser potential for future analysis. Then, the remaining peptides were again evaluated to select only the spots recognized with high affinity only by the pool of sera from dogs with CanL (asymptomatic and symptomatic). In addition, having low or no recognition with pools of non-infected canine sera (negative control), that is, serum from dogs healthy dogs and sera from dogs infected with *T. cruzi*, *Babesia* sp. and *Ehrlichia* sp.

4.4.7. Peptides synthesis

The selected peptides were synthesized in a solid phase, i.e., in a soluble form by the method developed by Merrifield (1969) in a 25 μmol scale, using the automatic synthesizer ResPep SL (Intavis1) (164). This synthesis method consists of fixing the C-terminal amino acid of the peptide on insoluble solid support and then extending the peptide chain by successive additions of residues from the C-terminal to the N-terminal portion. These amino acids have the amine group protected by the Fmoc group and a protective group to avoid unwanted reactions protected their side chain. The insoluble solid support is normally a resin, which was also protected by Fmoc. Rink Amide resin (Intavis) was used as a solid support. The protocol used for peptide formation was similar to the one used for synthesis in cellulose membranes. After the end of the last synthesis cycle, the peptide without the Fmoc group of the last amino acid w removed from the resin by a step called cleavage. In this step, the protective groups of the side chain were also removed. The synthesized peptides were solubilized, precipitated with cold methyl tert-butyl ether, and lyophilized. Then, the synthesized peptides went through production quality control to ensure that the peptides were synthesized correctly.

4.4.8. Confirmation of peptide identity by mass spectrometry (MALDI/TOF)

The synthesized peptides were submitted to the MALDI method (Matrix Assisted Laser Desorption/Ionization) that allows the ionization of several macromolecules combined with the TOF/MS/MS system (Time Of Fligh/Mass Spectrometer) (Bruker Daltonics) a kind of spectrometer of mass that utilizes the time-of-flight difference due to the size differences of the ionized substances.

The molecular weight of each peptide synthesized was confirmed by mass spectrometry using Autoflex Speed MALDI-TOF equipment. For the analysis, 0.5 µL of the concentrated sample was mixed with 0.25 mL of a saturated matrix solution 10 mg/mL α-cyano-4-hydroxycinnamic (Aldrich, Milwaukee, WI) in 50% acetonitrile/0.1% trifluoroacetic acid. The samples were applied to an MTP AnchorChip ™ 600/384 plate (Bruker Daltonics) and left to dry at room temperature. The raw data was obtained by MALDI-TOF/TOF Autoflex III™ (Bruker Daltonics, Billerica, USA) using a positive/reflector mode controlled by FlexControl™ 3.3 software. The instrument calibration was performed using reference peptides (Peptide Standard, Bruker Daltonics). Each spectrum was produced by accumulating data from 200 consecutive laser shots.

In addition to the MALDI-TOF/MS analyses, the synthetic peptides were subjected to serological ELISA assays using the same pools of sera previously used in the immunoblotting assays, in order to verify whether the results obtained in both assays corroborated. Soon after, confirming the identity of the synthesized peptides, they were used in a reactivity test against individual sera from dogs infected with *L. infantum*, asymptomatic and symptomatic, in addition, to control canine sera (non-infected, infected with *T. cruzi*, *Babesia* sp. and *Ehrlichia* sp.).

4.4.9. Preparation of crude extract of *L. infantum*

The total extract of the parasite was prepared from promastigotes of *L. infantum* (MHOM/BR/1974/PP75), which were kept at 24ºC in Schneider medium (Sigma-Aldrich, USA), supplemented with 10% inactivated fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Gibco/Thermo Fisher Scientific, USA). The parasites kept growing in the logarithmic phase by changing to new culture media every 3 to 4 days. Crude soluble antigen (CSA) was obtained from approximately 5x108 parasites that were washed and resuspended in 1 mL of 1X PBS, followed by lysis through 15 cycles of freezing in liquid nitrogen and thawing at 37°C. The concentration of total parasite proteins was quantified using the PierceTM BCA Protein Assay kit (Thermo Fisher Scientific, USA).

4.4.10. Validation of peptides in Enzyme-linked immunosorbent assay (ELISA)

Using the previously characterized individual samples from the canine serological panel, ELISA was utilized to evaluate the recognition pattern of specific IgG antibodies for the peptides and proteins. ELISA assays, using as antigens the recombinant proteins; KDDR-plus, Dyn-1, and K39 in addition to CSA and the peptides derived from the protein rKDDR-plus (K-plus 1 and K-plus 2) and protein rDyn-1 (Dyn-1 peptide) in addition a mixture of these three peptides (Dyn1, K-plus 1

and K-plus 2), were performed as previously described (20). Briefly, 96-well flat-bottomed microtiter plates (Costar) were coated with 0.1 µg/well of recombinant proteins and of the CSA and 0.3 µg/well of peptides for 16 hours at 37°C. Sera diluted 1:100 were added to wells in duplicates. Anti-dog IgG conjugated with horseradish peroxidase (Sigma) was used as secondary antibodies diluted 1:2500. A492 values were read in an automatic ELISA reader (SpectraMax 340 PC, Molecular Devices). The rDyn-1 and rKDDR-plus proteins from *L. infantum* were produced and purified as previously described (19, 20). Steven G. Reed (Infectious Disease Research Institute-IDRI, Seattle, Washington) kindly provided the rK39 antigen. The peptide mix was produced by mixing 0.3 µg of each target.

4.4.11. Statistical analysis

The ELISA results, recorded as optical density (OD) at 492 nm (Molecular Devices, USA), were stored and organized in Microsoft Excel 2010 spreadsheets. Subsequently, the data were distributed using scatter graphics computer software and statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Inc., USA). All serum samples were evaluated in duplicate, with the test result being the mean OD value of these simultaneous determinations. A receiver operating characteristic (ROC) curve was generated for each tested antigen considering the groups of negative dogs (*Babesia* sp., *Ehrlichia* sp., *T. cruzi*, and healthy dogs) and positive CanL (symptomatic, asymptomatic, and co-infected) applying the sensitivity values in the ordinate and the complement of specificity in the abscissa. Cut-off values were defined through of the curves for each antigen (by choosing the best compromise between sensitivity and specificity associated with the ROC curve), and values of sensitivity (165). For comparison of the OD between the antigens, all results were expressed by plotting the obtained values in an index format representative of the ratio between a given sample's OD and the cutoff value pertaining to each microplate, referred to as reactivity index (RI), with all results <1.00 considered negative and all results >1.00 considered positive. The ELISA results were compared with the serological status, previously defined by the work's reference methods (qPCR and parasitological). Each serodiagnosis test was evaluated with respect to sensitivity, specificity, area under the curve (AUC) considering a 95% confidence interval (95% CI), positive (PPV), and negative (NPV) predictive values, accuracy (AC) and likelihood ratio (LR) (166). The degree of agreement between the assays was determined by Kappa (κ) index calculated according to Cohen (167) and classified according to Landis and Koch (168) to assess the agreement between ELISA assays and reference methods (qPCR and parasitological): 0.00–0.20 (negligible agreement), 0.21–0.40 (weak), 0.41–0.60 (moderate), 0.61–0.80 (good) and 0.81–1 (excellent).

4.5. Results

4.5.1. Prediction of immunogenic epitopes of rDyn-1 and rKDDR-plus proteins

The complete protein sequence from rDyn-1 protein was used for predictions of B cell epitopes and structural disorder. Along the protein sequence, several epitopes with scores above the cut-off and with a high pattern of structural disorder were identified as illustrated in Figure 14 A. However, only sequences that had more than eight amino acids were considered potential targets and therefore were synthesized in the membrane. Thus, 17 peptide sequences were synthesized in the cellulose membrane as shown in Figure 14 B.

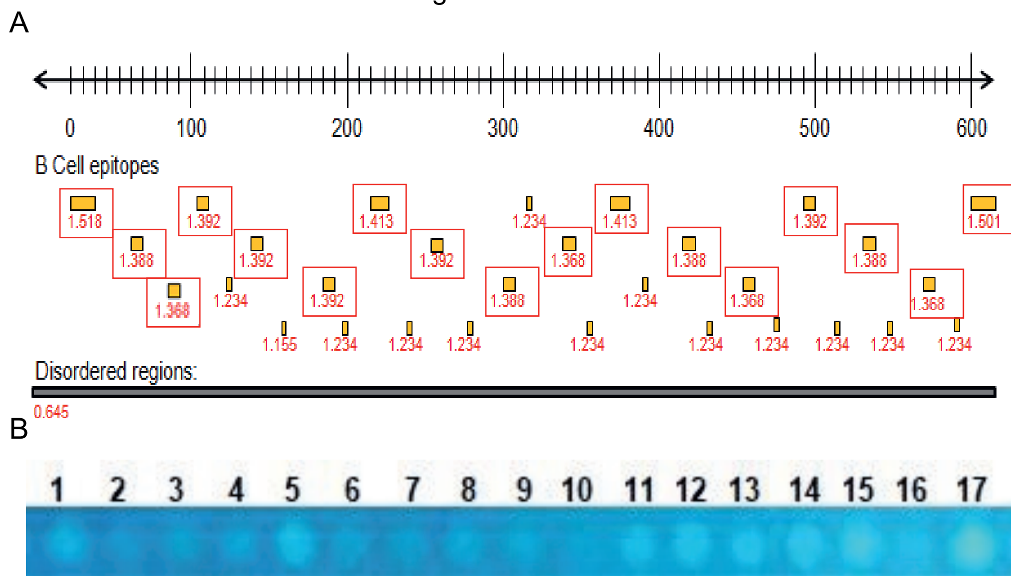

Figure 14 - Epitopes derived from the rDyn-1 protein. (A) Each point inside the red boxes represents a B-cell epitopes predicted by BepiPred defined as sequence regions with values above the threshold score of 0.6 and the numbers below indicate the prediction score obtained by each epitope. The black box indicates disordered regions predicted by IUPred with a score of 0.5. Predicted regions as B-cell linear epitopes that are associated with a high degree of structural disorder also exhibit a high antigenicity score, as observed in the graph at the center of the figure. (B) Cellulose membrane containing the 17 spots with the peptide sequences derived from the rDun-1 protein selected after bioinformatics analyses visualized by ultraviolet light.

For the identification of potential sequences derived from the complete rKDDR-plus protein, the repetitive block of 39 amino acids that compose the 15.3 sequences of the protein was used. Although the rKDDR-plus protein is characterized by the presence of a single repeating block, these repeats present degenerate regions in seven sites. Therefore, the amino acids that appeared more often in these

degenerate sites were defined to compose the sequence of 39 amino acids used as a template for the selection of peptide sequences (Figure 15).

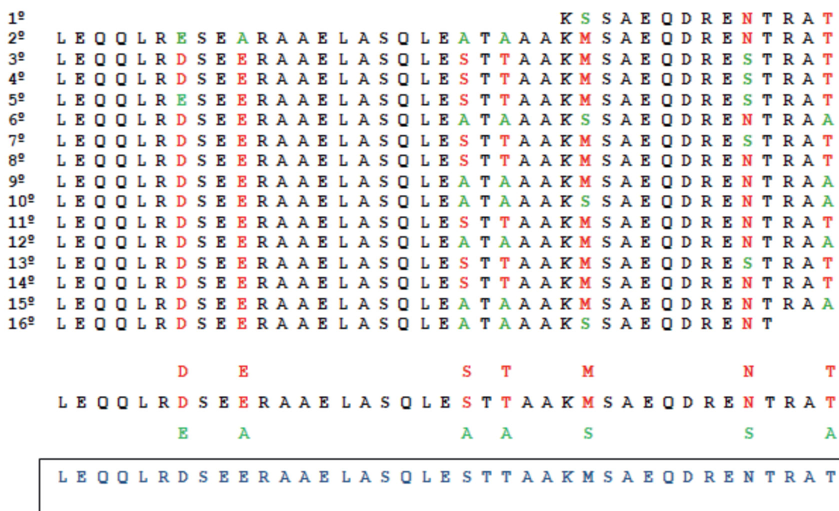

Figure 15 - Alignment of the repetitive array of amino acids that make up the rKDDR-plus protein. The 15.3 repetitive blocks containing the 39 amino acids were aligned for better visualization of the least repeated amino acids (green amino acid) and the most repeated (red amino acid) in the seven degenerate sites in black this sequence of the 39 amino acids that make up the 15.3 motifs of the rKDDR-plus protein. In blue is the final sequence used as a template used in the sliding window.

Subsequently, the template sequence was used based on a sliding window of 2 amino acids with a previously defined size that run through the template sequence to be encoded resulting in several smaller sequences of 10 amino acids. After the scan, 16 sequences were selected with the previously defined patterns; another four sequences containing 12 amino acids each were included for synthesis in order to cover a larger screening combination (Figure 16).

```
    L E Q Q L R D S E E R A A E L A S Q L E S T T A A K M S A E Q D R E N T R A T

 1  L E Q Q L R D S E E
 2      Q Q L R D S E E R A
 3          L R D S E E R A A E
 4              D S E E R A A E L A
 5                  E E R A A E L A S Q
 6                      R A A E L A S Q L E
 7                          A E L A S Q L E S T
 8                              L A S Q L E S T T A
 9                                  S Q L E S T T A A K
10                                      L E S T T A A K M S
11                                          S T T A A K M S A E
12                                              T A A K M S A E Q D
13                                                  A K M S A E Q D R E
14                                                      M S A E Q D R E N T
15                                                          A E Q D R E N T R A
16                                                            E Q D R E N T R A T
17  L E Q Q L R D S E E
18      Q Q L R D S E E R A
19          L R D S E E R A A E
20              D S E E R A A E L A
21                  E E R A A E L A S Q
22                      R A A E L A S Q L E
23                          A E L A S Q L E S T
24                              L A S Q L E S T T A
25                                  S Q L E S T T A A K
26                                      L E S T T A A K M S
27                                          S T T A A K M S A E
28                                              T A A K M S A E Q D
29                                                  A K M S A E Q D R E
30                                                      M S A E Q D R E N T
31                                                          A E Q D R E N T R A
32                                                            E Q D R E N T R A T
33  L E Q Q L R D S E E R A
34                          A E L A S Q L E S T T A
35                                                  A K M S A E Q D R E N T
36                                                        S A E Q D R E N T R A T
37  L E Q Q L R D S E E R A
38                          A E L A S Q L E S T T A
39                                                  A K M S A E Q D R E N T
40                                                        S A E Q D R E N T R A T
```

Figure 16 - Sliding window technique. The first ten amino acids of the template sequence formed the first set of oligopeptides. Then after a jump of 2 amino acids plus 10 amino acids were selected forming another set of oligopeptides. Following this logic, 16 oligopeptide sequences were selected. To complete the oligopeptide catalog 4 sequences composed of 12 amino acids with random sliding windows were included, completing the 20 sequences of oligopeptides that were synthesized on the membrane in duplicate.

Finally, 20 peptide sequences derived from the rKDDR-plus protein were elected for synthesis in duplicate, i.e., 40 peptides were synthesized on the membrane as illustrated in Figure 17.

Figure 17 - Cellulose membrane with peptide sequences derived from the rKDDR-plus protein visualized by ultraviolet light. Sixteen peptides selected after the scan were synthesized in sequence on the membrane (numbers 1 to 16 red), and then duplicates of these sequences were synthesized on the membrane (numbers 1 to 16 green). Then, the four sequences containing twelve amino acids each were included in the membrane (numbers 17 to 20 blue), followed by their duplicates (numbers 17 to 20 purple).

4.5.2. Immunoassay with membrane-bound peptides and densitometric analysis

Both the membrane containing the 17 peptides derived from the rDyn-1 protein, as well as the membrane containing the 40 derivatives of the rKDDR-plus protein were subjected to individual incubations on different days with the pool of sera from canines infected with *L. infantum* (asymptomatic and symptomatic) and not infected with *L. infantum* (healthy control and dogs infected with *T. cruzi*, *Babesia* sp. and *Ehrlichia* sp.) to evaluate the discrimination capacity of the selected peptides, through immunoblotting assays. After obtaining the chemiluminescence images of each assay, it was possible to observe points that stand out on the membrane, common in the different incubations, indicating recognition of the antibodies present in the pools used.

In the membrane that contained the epitopes derived from the rDyn-1 protein, although several peptides were shown to be reactive in the incubation with sera from symptomatic dogs, only one spot stood out in the incubation with asymptomatic sera. This same spot showed no reactivity against incubations with the sera of the control groups (healthy dogs, with *T. cruzi*, *Babesia* sp., and *Ehrlichia* sp.) proving to be a promising target to be soluble synthesized (Figure 18 A). Densitometry analyzes were performed to validate the selection of the peptide at position 10 (Dyn-1 peptide), as it presents a high recognition signal with the pool of sera from dogs infected with *L. infantum* (asymptomatic and symptomatic) and low recognition with sera from control dogs (dogs not infected with *L. infantum*, infected with *T. cruzi*, *Babesia* sp. and *Ehrlichia* sp.) (Figure 18 B).

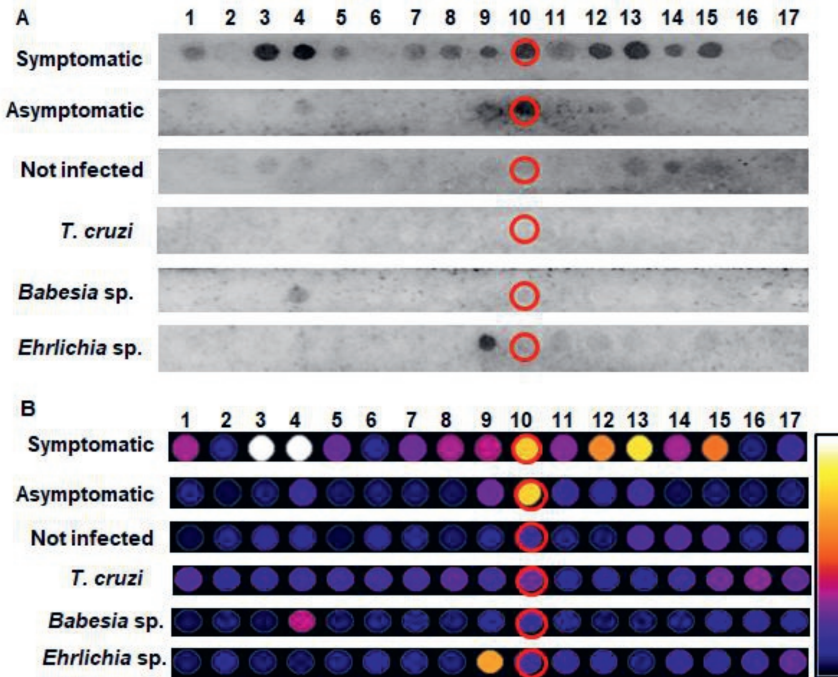

Figure 18 - Membrane immunoblotting analysis containing peptides derived from the rDyn-1 protein. (A) Chemiluminescence image from spot-synthesis technique to evaluate the reactivity and specificity of rDyn-1-derived peptides incubated with pools of canine sera from symptomatic and asymptomatic infected with *L. infantum* and sera from control dogs; healthy dogs without leishmaniasis other pathogens of interest (not infected), dogs infected with *Babesia* sp. and *Ehrlichia* sp. **(B)** Densitometry analysis of previously synthesized peptide spots on the cellulose membrane for selection of the most reactive spot in incubations with pools of dogs infected with *L. infantum*, not recognized by sera from dogs without CanL. The closer to the white hue of the scale, the more reactive the spot.

Twenty peptide sequences derived from the rKDDR-plus protein were screened by the scan. These sequences were synthesized in duplicate to reinforce the immunoblotting results. Therefore, it is possible to observe 40 peptides on the membrane, facilitating the identification and localization of duplicated peptides. From the images obtained from the chemiluminescence of the membrane immunoblotting (Figure19 A), it is possible to observe that none of the spots showed reactivity during incubation with the control sera pools (healthy dogs, *T. cruzi*, *Babesia* sp., or *Ehrlichia* sp.). This result indicates that the peptides do not present cross-reactivity with other evaluated parasites or even with sera from healthy dogs. On the other hand, when incubating the membrane with pools of sera with *L. infantum*, some spots and its duplicates were reactive for both clinical forms. Spots 13 and 29 (duplicate) with red circle, spots 35 and 39 (duplicate) with green circle, and spots 36 and 40 (duplicate) with yellow circle were clearly visible during incubation with the symptomatic pool of

sera and were slightly reactive during incubation with the pool of asymptomatic sera. Spot 14 (dark blue) and its duplicate (spot 30) were strongly recognized by the symptomatic pool and did not light up when incubated with an asymptomatic pool. When analyzing the membrane incubated with the asymptomatic pool spots 5 and 21 (duplicate) with light blue circle, were recognized, but did not show any reactivity with the pool of symptomatic sera. However, after normalizing the duplicates of the spots and analyzing the scores obtained in the densitometry analyses (Figure 19 B), spots 35 and 39 (duplicate) with green circle were selected, because they presented the highest recognition signal with both sera from symptomatic dogs and sera from asymptomatic dogs. In addition, spots 5 and 21 (duplicate) with light blue circle also were selected because it showed better recognition signal with antibodies from asymptomatic dogs. The other spots were not selected for synthesis in this work, because although they were recognized by the antibodies present in the sera pools of dogs with *L. infantum*, their scores were lower.

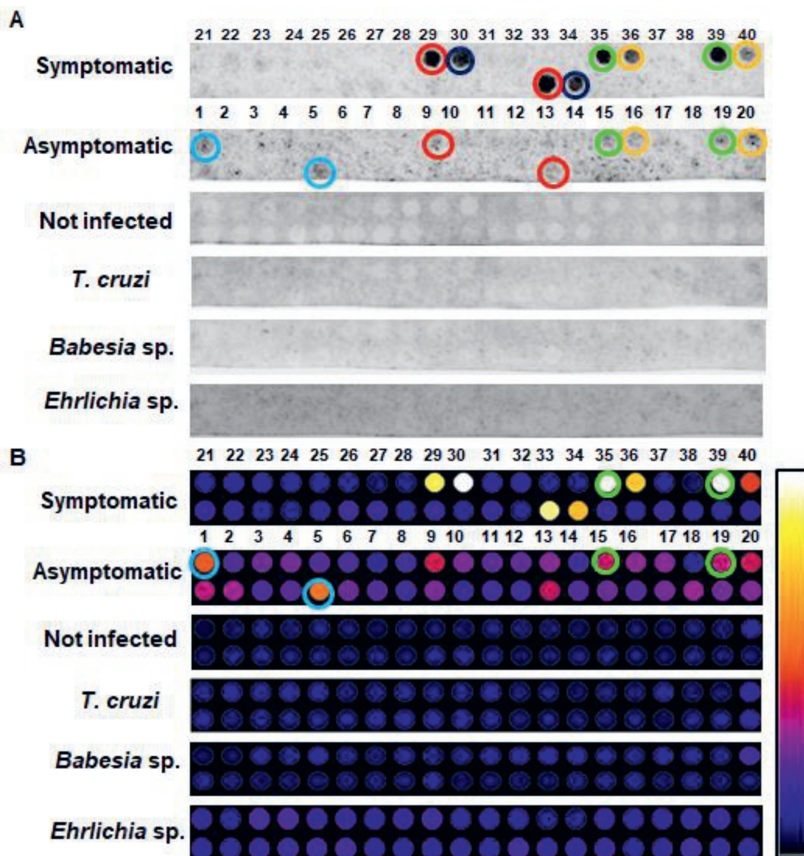

Figure 19 - Analysis of the membrane containing peptides derived from the rKDDR-plus protein. (A) Chemiluminescence image from membrane immunoblotting assays incubated with pools of sera from symptomatic and asymptomatic dogs infected with *L. infantum* and sera from control dogs; healthy dogs

without leishmaniasis other pathogens of interest (not infected), dogs infected with *Babesia* sp. and *Ehrlichia* sp. Red, green, and yellow spots were lit for both groups of infected dogs. Dark blue spot lit only for the pool of symptomatic dogs and light blue spot lit only for the pool of asymptomatic sera. **(B)** Densitometric analysis for selection of the most reactive spots in incubations with pools of dogs infected with *L. infantum*, not recognized by sera from dogs without CanL. The closer to the white hue of the scale, the more reactive the spot. Green circle; reactive spots with symptomatic and asymptomatic sera. Blue circle; more reactive spots only with asymptomatic sera.

4.5.3. Soluble synthesis of peptides and mass spectrometry (MALDI/TOF)

After immunoblotting and densitometry analysis, three peptide sequences were synthesized in the solid phase (SPFS) as described in the Table 8. The peptides were produced anchored to polymeric support that allowed their production in soluble format (164).

Table 8 - Selected peptic sequences.

Peptide	Predicted molecular mass (g/mol)	Molecular mass MALDI TOF/MS/MS (g/mol)	Sequence	Source protein
Dyn-1	1201.29	1201.6876	I-K-R-D-D-R-K-D-D	rDyn-1
K-plus 1	1377.63	1378.6980	A-K-M-S-A-E-Q-D-R-E-N-T	rKDDR-plus
K-plus 2	1101.54	1102.6672	E-E-R-A-A-E-L-A-S-Q	rKDDR-plus

To confirm whether the synthesized soluble peptides were compatible with the expected sequences, the crude products were subjected to mass spectrometry analysis by MALDI in TOF/MS/MS as shown in Figure 20 mode, which confirmed the expected molecular mass and, therefore, the compatibility with the expected peptide sequences.

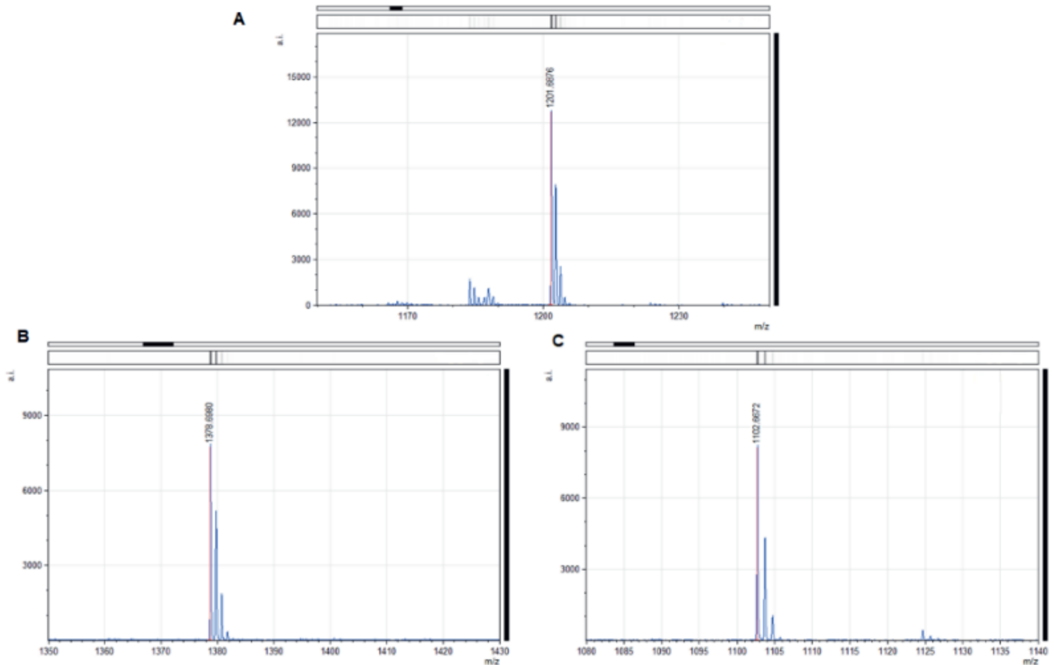

Figure 20 - MALDI-TOF MS peptide elution analysis. (A) MALDI-TOF MS analysis of the peak from Dyn-1 peptide. (B) MALDI-TOF MS analysis of the peak from K-plus 1. (C) MALDI-TOF MS analysis of the peak from K-plus 2.

4.5.4. Comparative efficacy between rDyn-1 and KDDR-plus precursor proteins and their more immunogenic peptide derivatives

ELISA experiments were performed using the complete recombinant proteins Dyn-1 and KDDR-plus, the synthetic peptides derived from each recombinant protein (Dyn-1, K-plus 1, and K-plus 2 peptides), and control antigens rK39 and CSA against a canine serological panel to identify which of them offered highest performances. Furthermore, a pool containing a mixture (Mix peptides) of 0.3 µg/well of each peptides evaluated in this work was also included. Figure 21 illustrates the performance parameters and reactivity index (RI) distributions obtained by ELISA tests using the recombinant proteins, peptides and control antigens performed on 119 samples from dogs with CanL being 50 symptomatic dogs, 50 asymptomatic and 19 co-infected (*Leishmania* and *Babesia* sp.); 33 dogs with other infections (*Babesia* sp., *Ehrlichia* sp. and *T. cruzi*) and 34 samples from not infected dogs.

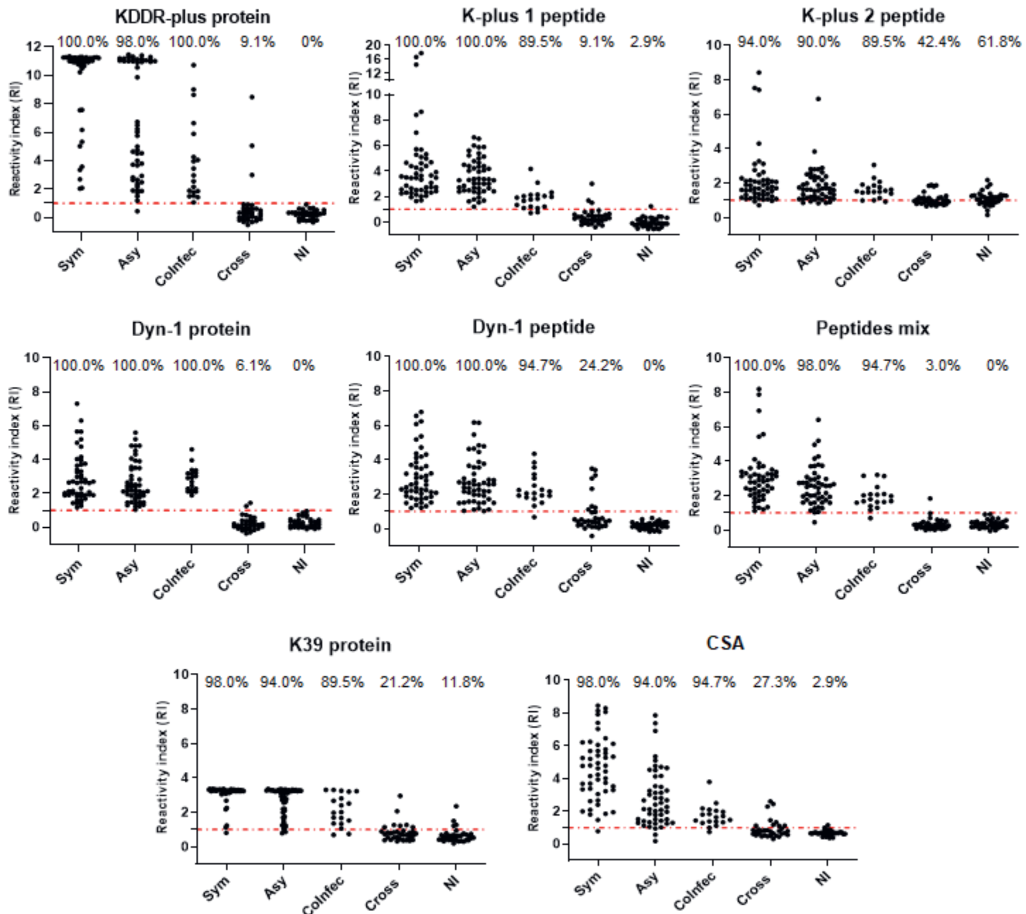

Figure 21 - Comparing the reactivity of canine sera using rKDDR-plus, rDyn-1 and K39 proteins, their derived peptides K-plus 1, K-plus 2, Dyn-1 and mixtures of these three peptides and the crude extract of *L. infantum* in an ELISA protocol. Reactivity index results obtained under *Leishmania* complete recombinant proteins Dyn-1 and KDDR-plus, the synthetic peptides derived from each recombinant protein (Dyn-1, K-plus 1 and K-plus 2 peptides), a mixture (Mix peptides) of 0.3 µg/well of the three peptides and control antigens rK39 and CSA against a canine serological panel composed by serum from infected dogs presenting symptoms (Sym), infected asymptomatic dogs (Asy), dogs co-infected with *Leishmania* and *Babesia* sp. (CoInfec), dogs with other infections (*Babesia* sp., *Ehrlichia* sp. and *T. cruzi*) (Cross) and not infected (NI). The index above each column in the plot indicates the percentage of samples that are above the cut-off. Abbreviation: (CSA) crude soluble antigen.

The diagnostic performance of each antigen evaluated was summarized in table 9. rDyn-1 protein was the only antigen able to determine all cases of dogs

carrying *L. infantum* (asymptomatic, symptomatic and co-infected) yielding an overall sensitivity of 100%. The mixture of peptides, the Dyn-1 and K-plus 1 peptides and the rKDDR-plus protein were able to identify 100% of the dogs considered symptomatic, but failed to identify asymptomatic or co-infected dogs. Thus, the general sensitivity of the rKDDR-plus protein and the Dyn-1 peptide was 99.2% and the peptide mixture and the K-plus 1 peptide was 98.3%. The other antigens were not able to determine with 100% none of the groups of infected dogs. Despite not having recognized all dogs positive for CanL, the peptide mixture showed the best specificity result = 98.5% in addition to the highest LR value (65.87) among all the antigens evaluated in this work. The proteins rDyn -1 and rKDDR-plus followed by peptide K-plus 1 also showed satisfactory results in relation to specificity 97.0%, 95.5% and 94.0%, respectively, and LR 33.5, 22.2 and 16.5, respectively. Based on these results, it is possible to suggest the use of a mixture of peptides in addition to K-plus 1 and Dyn-1 peptides as new markers for the diagnosis of CanL.

Table 9 - Serological evaluation of antigens for diagnosis of leishmaniasis.

Antigen	**Sensitivity (N = 119) [CI 95%]**	**Sensitivity (%)**			**Specificity (N = 67) [CI 95%]**	**Specificity (%)**			
		Sym. (N = 50)	**Asy. (N = 50)**	**CoInfec (N = 19)**		**Bab. (N = 7)**	***Ehr.* (N = 6)**	***T. cruzi* (N = 20)**	**NI (N = 34)**
rDyn-1 protein	100 [97.0 – 100	100	100	100	97.0 [89.6 – 99.7]	71.4	100	100	100
Mix peptides	98.3 [94.1- 99.8]	100	98.0	94.7	98.5 [92.0 – 100]	100	83.3	100	100
rKDDR-plus protein	99.16 [95.5 – 100]	100	98.0	100	95.5 [87.5 - 99.1]	71.4	100.0	95.0	100
K-plus 1 peptide	98.3 [94.1 – 99.8]	100	100	89.5	94.0 [85.4 – 98.4]	100	66.7	95.0	97.1
Dyn-1 peptide	99.16 [95.4 – 100]	100	100	94.7	88.0 [77.8 – 94.7]	100	100	60.0	100
CSA	95.8 [90.5 – 98.6]	98.0	94.0	94.7	85.0 [74.3 – 92.6]	71.4	100	65.0	97.1
rK39	94.96 [89.4 – 98.1]	98.0	94.0	89.5	83.6 [72.5 – 91.5]	71.4	66.6	85.0	88.3
K-plus 2 peptide	91.6 [85.1 – 95.9]	94.0	90.0	89.5	47.8 [35.4 – 60.3]	57.1	33.3	65.0	38.2

Abbreviations: CSA: crude soluble antigen, CI: confidence interval, Sym.: symptomatic, Asy.: asymptomatic, CoInfec: co-infected, Bab.: *Babesia* sp., Ehr.: *Ehrlichia* sp, NI: not infected, LR: likelihood.

ROC curves were generated for each antigen tested to determine the test sensitivity and specificity (Figure 22). Analysis of the area under the curve (AUC) confirmed the superior performance of the rDyn-1 protein and the peptides mixture (AUC = 0.9982 and 0.9934, respectively) in comparison to the other proteins and peptides evaluated; K-plus 1 peptide (AUC = 0.9888), rKDDR-plus protein (AUC =

0.9823), rK39 (AUC = 0.9686), CSA (AUC = 0.9536), Dyn-1 peptide (AUC = 0.9482) and K-plus 2 peptide (AUC = 0.8243).

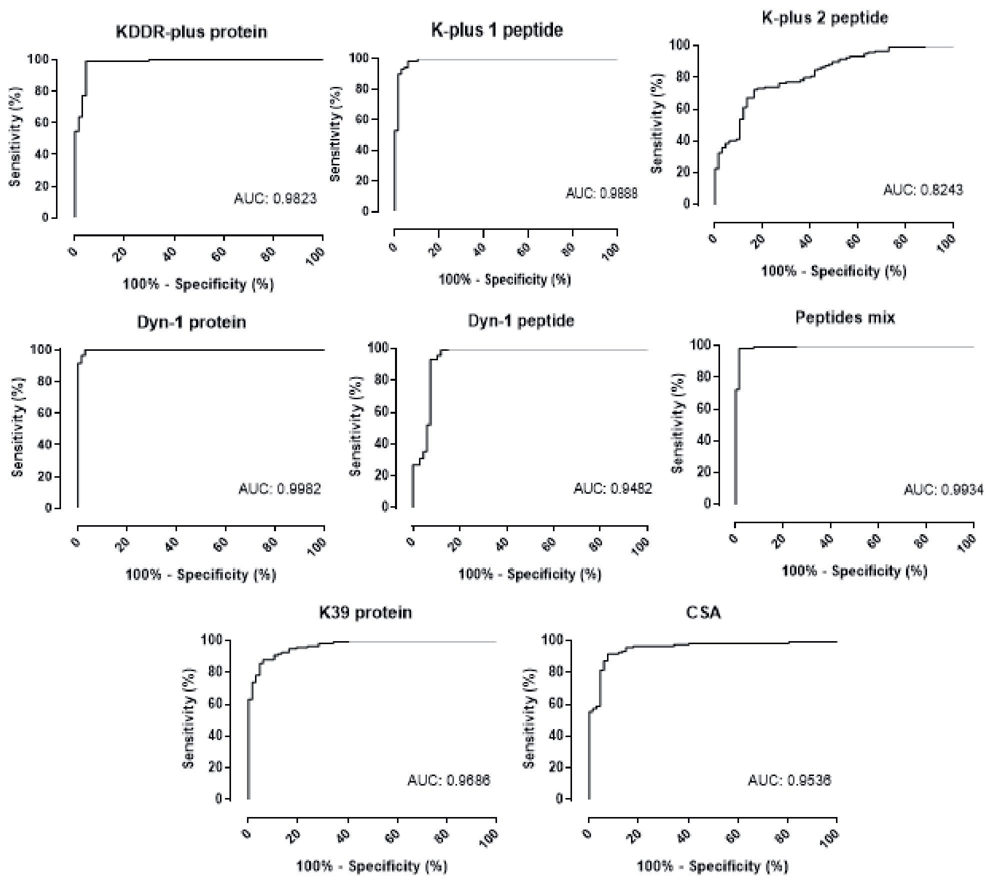

Figure 22 - ROC curve analysis of the area under the curve (AUC), considering the results from ELISA. Comparation of the ROC curve obtained employing ***Leishmania*** complete recombinant proteins Dyn-1 and KDDR-plus, the synthetic peptides derived from each recombinant protein (Dyn-1, K-plus 1 and K-plus 2 peptides), a mixture (Mix peptides) of 0.3 µg/well of the three peptides and control antigens rK39 and CSA was established by GraphPad Prism 8.0 using serum samples of negative and positive samples in each plate. Abbreviations: (AUC) area under curve; CSA crude extract.

The results obtained in ELISA tests confirmed that the peptide mixture in the rDyn-1 protein and peptide mixture presented the best diagnostic performances for CanL. These results were confirmed by the Kappa agreement index of 0.9977 (rDyn-1 protein) and 0.965 (mix peptides) with a level of agreement excellent. In addition, the rKDDR-plus protein and its derived peptide K-plus 1 also appear favorable, with a Kappa index > 0.9 and a degree of agreement considered excellent. Although the

Dyn-1 peptide and the CSA also showed a degree of agreement considered excellent, their Kappa values were less than 0.9. The rK39 protein and the K-plus 2 peptide, on the other hand, presented the lowest Kappa values and, consequently, the lowest agreement levels among the analyzed antigens. It is noteworthy that both the precursor proteins rDyn-1 and rKDDR-plus and the peptides derived from them, K-plus 1 and Dyn-1, together with the mixture of peptides proved to be good targets to be used in the diagnosis of CanL. The rDyn-1 protein showed the best performance in CanL diagnosis (98.9% accuracy), followed the peptide mixture (98.4% accuracy), the rKDDR-plus protein (97.9%), by the K-plus 1 and Dyn-1 peptides (accuracy of 96.8% and 95.2%, respectively), followed by the control antigens CSA and rK39 (accuracy of 91.9% and 90.9%, respectively) and finally by the K-plus 2 peptide (accuracy of 75.8%) (Table 10).

Table 10 - Comparison of performance of diagnostic tests for CanL using a validation serum panel.

Positive samples			Negative samples				PPV (%)	NPV (%)	AC (%)	K	Level of Agreement
Sym.	Asy.	CoInfec	Bab.	Ehr.	*T. cruzi*	NI					
50/50	50/50	19/19	5/7	6/6	20/20	34/34	98.3	100	98.9	0.977	Excellent
50/50	49/50	18/19	7/7	5/6	20/20	34/34	99.2	97.1	98.4	0.965	Excellent
50/50	49/50	19/19	5/7	6/6	19/20	34/34	97.5	98.5	97.9	0.953	Excellent
50/50	50/50	17/19	7/7	4/6	19/20	33/34	96.7	96.9	96.8	0.930	Excellent
50/50	50/50	18/19	7/7	6/6	12/20	34/34	93.7	98.3	95.2	0.893	Excellent
49/50	47/50	18/19	5/7	6/6	13/20	33/34	91.9	91.9	91.9	0.822	Excellent
49/50	47/50	17/19	5/7	4/6	17/20	30/34	91.1	90.3	90.9	0.798	Good
47/50	45/50	17/19	4/7	2/6	13/20	13/34	75.7	76.2	75.8	0.429	Moderate

ns: CSA: crude soluble antigen, Sym.: symptomatic, Asy.: asymptomatic, CoInfec.: co-infected, Bab.: , Ehr.: *Ehrlichia* sp, NI: not infected, PPV: positive predictive value, NPV: negative predictive value, AC: .: index kappa.

4.6. Discussion

In recent years, there has been a steady increase in studies aimed at finding a more accurate diagnosis for CanL. A considerable part of these studies, if not the majority, was focused on the identification of highly immunogenic molecules to the antigens of the parasite responsible for the disease. In this context, highly sensitive and specific antigens, capable of being used in simple, rapid tests and with easy adaptability in the field, dispensing with the use of expensive equipment and specialized technicians, were at the center of these discussions.

Recombinant protein production technology is a tool that has helped a lot in the search for new antigens for both leishmaniasis and other diseases (169). In the previous chapters, it is demonstrated that recombinant antigens from *L. infantum* have high sensitivity and specificity in the diagnosis of CanL, especially in dogs without clinical signs of the disease, asymptomatic ones (123, 160). The identification of infected dogs, regardless of clinical status, is necessary as part of the disease prevention and control measures in the canine population and consequently in the human population.

In the previous chapters it is also shown that rKDDR-plus and rDny-1 proteins carry immunodominant epitopes, which may be useful for the serological diagnosis of CanL. The results of screening a panel of dog sera using immunoblotting assays strongly suggest that sequences derived from rDyn-1 and rKDDR-plus would be useful for developing antigens that could improve the sensitivity and specificity of diagnostic tests currently available. Therefore, in this chapter, the performance of three synthetic peptides and a mixture of these derivative peptides from previously described and characterized antigens for use in the diagnosis of CanL was evaluated.

The rKDDR-plus, which proved to be advantageous in terms of specificity, performed in an excellent manner to discriminate dogs without infection by *Leishmania*, i.e. healthy dogs or dogs that have a pathology other than leishmaniasis that could cause a false positive result (123). Corroborating the findings of Chapter 2, the results in this chapter show a low cross-reactivity index of rKDDR-plus, maintaining the ability to identify dogs with CanL. One of the peptides derived from rKDDR-plus, the K-plus 1 peptide, presented a diagnostic performance similar to its precursor with good sensitivity of 98.3% (K-plus 1) and 99.2% (rKDDR-plus) and specificity 94.0% (K-plus 1) and 95.5% (rKDDR-plus). On the other hand, the second peptide derived from rKDDR-plus (K-plus 2) obtained a lower than expected diagnostic quality. Its diagnostic accuracy was only 75.8%, below all the antigens evaluated in this work, including the CSA control antigens (91.9% accuracy) and rK39 (90.9% accuracy), presenting results that indicate that it is not a good diagnostic marker for CanL when used individually. This shows that individual peptides sometimes can exhibit mixed performance results. However, when used together and in previously studied proportions, they can reach an accuracy equal to or greater than the sum of the individualized peptides.

Another protein used in this chapter for peptide selection was the Dyn-1 recombinant protein. In previous chapters, it showed a satisfactory performance regarding rDyn-1, a protein belonging to the protein superfamily dynamins related to several processes of membrane dynamics and functioning, for the diagnosis of CanL (160). The rDyn-1 protein stood out for its excellent ability to identify dogs with leishmaniasis, especially dogs without clinical signs compatible with the disease, and that present positive diagnostic in a combination of tests (serological, molecular, and/or parasitological) characterized as asymptomatic. Among all the antigens (proteins, peptides, and CSA) evaluated in this chapter, the rDyn-1 protein showed the best diagnostic accuracy (98.9%) maintaining the high ability to identify all dogs with CanL (symptomatic, asymptomatic, and co-infected) (sensitivity = 100%) corroborating with the previous studies. Besides presenting a low cross-reaction index with dogs without CanL or with other diseases (specificity = 97.0%). Here only one

peptide derived from rDyn-1 protein (Dyn-1 peptide) was selected to be tested in the ELISA assays. This peptide was the only one that was shown to be reactive in the incubation with the pool of sera from dogs infected with *L. infantum* (asymptomatic and symptomatic) presenting low or no degree of recognition with sera from control dogs (dogs not infected with *L. infantum*, infected with *T. cruzi*, *Babesia* sp. and *Ehrlichia* sp.) in the immunoblotting assay. As well as in the immunoblotting assays, the Dyn-1 peptide was also able to identify all symptomatic and asymptomatic dogs in ELISA assays, but it was not able to determine all co-infections (*Leishmania* and *Babesia* sp.) presenting a sensibility de 99.2% and a moderate index of cross-reaction with dogs with *T. cruzi* (specificity = 88.1%).

Additionally, a fourth peptide, composed of a homogeneous mixture of the three peptides (Dyn1, K-plus 1, and K-plus 2) evaluated here, was included for analysis in ELISA assays. The peptide mix, surprisingly, showed excellent diagnostic performance, similarly to the rDyn-1 protein. Although the rDyn-1 protein showed the best accuracy (98.9%) and sensitivity (100%) among all the antigens analyzed in this study, the peptides mix showed the highest specificity values (98.5%) and likelihood ratio (65.9) as compared to the rDyn-1 protein (specificity = 97.0 and likelihood ratio 33.5). This result shows the potential of this mixture as a serological marker for the diagnosis of CanL. Although the performance of the K-plus two peptide individually was lower than expected, when present in the peptide mixture, the performance of this mixture was attractive. This result reinforces the theory that antigenic mixtures with multiepitopes or chimeric character may be the way to achieve impeccable diagnostic performance.

Peptide synthesis is another recently introduced alternative for researchers to search for antigenic targets for the disease. Synthetic peptides are excellent antigens to be used in several serological tests, including ELISA. These small portions are designed from amino acid sequences of potentially antigenic proteins already characterized for the diagnosis of the disease. Synthetic peptides have an advantage over proteins because the fragments are selected based on a specific criterion or characteristic. Therefore, these sequences are composed of epitopes that are more singular and directed to the target of interest. With this, it is possible to build a larger molecule, composed of different sequences with specific characteristics that, when used together, provide higher diagnostic accuracy than that obtained with their individual use. This approach has been gaining strength in the diagnosis of CanL with the use of chimeric antigens or antigenic mixtures composed of multiple epitopes to be used as antigens in serological tests (170).

It is noteworthy that in this chapter the traditional rK39 and CSA antigens were included as a control because they are widely used in the serological diagnosis of CanL in Brazil and worldwide. However, using the total extract of the parasite has fallen into disuse in view of its high rates of cross-reactivity reactions with other pathogens that often coexist in endemic areas, leading to inconsistent results (171, 172). In turn, the limitation of the rK39 antigen was mainly related to the highly varied performance between different regions of the world, making its use limited to geographic regions where previous studies have shown good performance (66, 117). As reported in the literature, the CSA and rK39 antigens showed limited results for specificity (85.1% and 83.6%, respectively) resulting in an accuracy of 91.9% and

90.9%, respectively increasingly discouraging the use these targets as markers for the diagnosis of CanL.

In the search for targets that help in the diagnosis of diseases such as leishmaniasis, many works have focused on a deeper study of genes and their transcripts and protein products involved in important cellular processes, as in the case of the two recombinant proteins and their peptides used in this work. The search for new antigens is a constant search, which must be done to reach a diagnosis that presents 100% sensitivity and specificity. With such antigens, it is expected that the development of more accurate diagnostic tests, which present sufficient sensitivity to correctly identify all cases of leishmaniasis. In addition, they would have satisfactory specificity, allowing the correct discrimination of case negatives avoiding false positive results. It is important to remember that the early diagnosis of CanL can help in the control of the infection in dogs and, consequently, in the reduction of human infection.

Based on the results obtained so far, it is expected to contribute to the control of leishmaniasis developing tests that are more efficient and encouraging the production of new technologies both for the diagnosis of leishmaniasis and for other infectious diseases.

4.7. Conclusion

Although the diagnosis of CanL still faces serious difficulties, this chapter shows that both the rDyn-1 protein and the peptide mix (peptides mix) present a high performance and can be applied as markers for the diagnosis of CanL. However, tests with a broader sample bank or a multicenter study with representative samples from different regions of Brazil is necessary to confirm the results obtained here. Furthermore, testing with different concentrations and compositions of the peptide mix or even the construction of a chimera may be relevant.

4.8. Transition

In this chapter, a more detailed screening of the amino acid sequences of the whole proteins that were the center of discussions in Chapters 2 and 3 of this thesis was carried out. In order to identify peptide sequences responsible for the excellent performance of both proteins, initial *in silico* screenings (bioinformatics analysis) with subsequent *in situ* validation (immunoblotting and ELISA) to form a functional multi-epitope for application in the diagnosis of CanL were performed. The rationale for the study around the rDyn-1 protein was to locate the immunogenic epitopes responsible for its excellent performance in the identification of dogs without clinical signs of leishmaniasis. On the other hand, for rKDDR-plus, the search was focused on identifying peptide sequences related to the ability to identify dogs with *Leishmania*, showing the lowest cross-reactivity index with other pathologies. As a result, a peptide derived from the rDyn-1 protein was selected for presenting the best likelihood value in ELISA assays among all the antigens evaluated in this chapter. Furthermore, a mixture of peptides selected in this work also presented interesting results, reinforcing the theory that antigenic mixtures with multi-epitopes or chimeric may be the way to

achieve impeccable diagnostic performance. However, more specific validations with better defined criteria should be considered to fully explore the potential of peptides, even those that performed below expectations when evaluated individually.

In parallel to this research, a thermal biosensor platform was developed that has previously shown to be useful for the point-of-care detection of glucose or bacterial infection (173-176). In Phase II of the research collaboration, the protein targets identified in this thesis will be immobilized on aluminum chips for thermal rebinding analysis. The resulting biosensor platform will not only allow for the qualitative detection of VL antibodies but will also allow quantifying the response. Furthermore, due to the sensitivity of the platform, it is hypothesized to outperform low-cost serological tests in the detection of trace amounts of antibodies. This might contribute even further to the performance of the serological test in correctly identifying asymptomatic dogs with VL.

5. Conclusion and final thoughts

The main objective of the thesis was the identification and selection of amino acid sequences that presented an excellent response against antibodies from individuals or dogs carrying *Leishmania*. Such sequences were essential for the final design and synthesis of an antigen composed of multi-epitopes for application in the serological diagnosis of leishmaniasis and biosensor. This objective was in line with one of the leishmaniasis control measures in Brazil and some other parts of the world. These measures focus on early diagnosis of canine and human cases to contain the disease and minimize further spread. An accurate, quick and easy diagnosis can therefore contribute to reducing the global incidence of the disease.

Serological methods (ELISA and ICT) are important allies in the diagnosis of leishmaniasis and in this thesis; they were used to validate new antigenic targets for the diagnosis of the disease. In Chapter 1, important concepts about the disease were presented to the reader, exposing a brief history of how its diagnosis was performed over several years doing an extensive literature study to understand the most recent advances in the diagnosis of the disease, their potential impact in this field, and their shortcomings.

With the information in Chapter 1, the advantages of serological techniques such as ELISA and ICT, for the diagnosis of the disease could be seen. In turn, these techniques were used in the second chapter of this thesis to validate the rKDDR-plus protein as an antigen for LVH and CanL serodiagnosis. The second chapter explored the kinesin protein superfamily related to the rK39 protein, a precursor for the use of recombinant antigens in the diagnosis of leishmaniasis. A careful study along the complete kinesin sequence enabled the discovery of rKDDR-plus, which has shown more promise in the diagnosis of leishmaniasis. However, in order to add more value to the diagnosis of the disease, the next studies went towards searching for targets capable of identifying clinically healthy but infected dogs. These asymptomatic dogs do not show any clinical signs on physical examination or any pathological clinical abnormalities but are still infected by *Leishmania*. Therefore, this category of dogs is hard to identify using classical methods and as a result, these dogs can freely spread the disease throughout the population further complicating containment. Unfortunately, modern tests like the serological assays based on rKDDR-plus presented in Chapter 2, still struggle with correctly identifying these dogs. Therefore, the search for alternative antigens and peptides continued and a second protein superfamily of *Leishmania* was studied in this work, i.e. dynamins.

Until this thesis research, the rDyn-1 protein, derived from the dynamin protein superfamily, had never been related in any study to the identification of leishmaniasis. However, after *in silico* bioinformatics analysis that highlighted this family as potentially interesting for this purpose this protein was selected. The results in Chapter 3, illustrate the excellent ability of this antigen to identify the most important group of *Leishmania*-infected dogs, the asymptomatic ones, through ELISA.

Having in hand two new antigens with potential for the diagnosis of leishmaniasis, the following efforts were to be directed towards the identification of the peptide sequences present in both rDyn-1 and rKDDR-plus that make these antigens so useful in detecting this disease. This understanding could then be used

to design new antigens composed only of these specific sequences. This was the rationale behind the research presented in Chapter 4.

Chapter 4 aimed to carefully analyze the complete protein sequences of rKDDR-plus and rDyn-1. The objective was to identify and select the peptides present in the rDyn-1 protein responsible for the identification of asymptomatic dogs. Moreover, research focused on the identification of the peptides present in the rKDDR-plus protein responsible for the ability to discriminate *Leishmania*-infected dogs from non-infected dogs, without presenting cross-reacting with other organisms. To achieve that goal, bioinformatics tools were used for the selection of hundreds of peptides that were later screened in immunoblotting assays for the selection of single peptides. This approach finally resulted in a peptide sequence derived from rDyn-1 and two derivatives of rKDDR-plus. Although the hypothesis was that these peptides would already perform well in identifying the infection, the research was taken a step further by using a proportional mixture of the three peptides for the canine serological tests. This resulted in a study in which whole recombinant proteins as well as their more antigenic fragments had their performances serologically evaluated and compared. The results obtained reinforce the theory that sometimes individual peptides can present low-performance results but when used together and in carefully studied proportions, they can reach accuracy greater than the sum of the individualized peptides.

In summary, the biomarkers currently used in the diagnosis of VL are whole proteins or pieces of proteins. These biomarkers are macro-molecules composed of sequences that may or may not be involved in the antigen-antibody relationship. The production of recombinant proteins can be said to have revolutionized serological diagnostic methods for infectious diseases and are now in widespread use. In fact, these proteins are of great importance for diagnostic evolution, however, the need to produce, prepare, and purify the antigens, together with the respective quality control must be considered. On the other hand, short peptides can be automatically synthesized and produced cheaply in large quantities and are chemically more stable and capable of long-term storage, making their use more advantageous. Therefore, in the last chapter of this thesis, the focus was exclusively on identifying shorter portions present in the entire proteins that were presented in chapters 2 and 3. These proteins were sought for smaller fragments that were directly related to the characteristics that made them relevant to the diagnosis of leishmaniasis. Therefore, the future intention of this work is to combine the best evaluated peptide sequences in a new molecule composed only of specific portions of these proteins. Because, short peptides that cover linear epitopes of B cells replace the use of whole proteins, avoiding the display of unnecessary sequences. Thus, the number of false positive results decreases, thus improving diagnostic specificity. On the other hand, the sensitivity of the test can also be improved by combining fragments of peptides that present a greater amount of immunoreactive epitopes that are more strongly presented. Thus, by creating a molecule composed of multiple antigenic peptides, it is possible to improve the accuracy of diagnostic tools for leishmaniasis and other infectious diseases.

However, every effort to identify more sensitive and specific molecules will only make sense if these molecules can actually be incorporated into diagnostic tools

that can be introduced into disease control programs. Therefore, the findings of this thesis will be valid in two platforms for diagnosing the disease; an immunochromatographic device (ICT), already used as a diagnostic tool for VL, and a biosensor, an unprecedented tool for diagnosing the disease. The ICT test is being prepared in partnership with Safe Test Diagnostics/Brazil, the same company that owns the ICT/KDDR-plus production and marketing rights. Confirmation of the diagnostic capacity of the molecule described in this thesis in this rapid test format is expected soon.

The molecule described in this thesis will also be validated in a biosensor format. However, it is important to emphasize that the findings of this thesis are only the initial part of a global work that involves a partnership between two internationally renowned institutions, the Maastricht University, Netherlands, and the Federal University of Minas Gerais (UFMG), Brazil. The first phase carried out at UFMG ended with the identification of potential peptides that will form a multi-peptide molecule to be used in a new diagnostic tool for leishmaniasis, the biosensor. The introduction of this molecule in the biosensor is part of the second phase of this work, which will continue at the Sensor Engineering Department of Maastricht University after this project. The results presented here, therefore, provide a new and robust multi-epitope formulation as a basis for the development of improved peptide-based diagnostic tools for leishmaniasis. This thesis is part of a larger trend in the current field of parasitology research in which bioinformatics tools are combined with immunological techniques for the identification, selection, and evaluation of relevant molecules, in addition to seeking new tools so that these molecules can be applied. Thus, this work contributed to new advances in this area by identifying promising targets and seeking new devices for the use of this molecule. In this way, it is expected that when validating a biosensor, which is low cost and easy to perform for the diagnosis of leishmaniasis, this fact will open new premises for the improvement of the diagnosis of other parasitic infections as neglected as leishmaniasis, mainly in countries of low income.

6. Abstract

Visceral leishmaniasis (VL), also known as kala-azar, is a systemic infectious disease with a chronic course, characterized by long feverish periods, weight loss, anemia and changes in internal organs such as the spleen and liver. Such changes lead to a progressive weakness of the individual that can progress to death when not treated early. VL is endemic in several countries and represents a serious public health problem with a high incidence.

In the Americas and southern Europe, VL is zoonotic, with dogs being its main reservoir in urban areas. In these regions, VL is considered a disease of great human and veterinary medical importance. In Brazil, the disease is endemic and is expanding rapidly into municipalities that have not yet diagnosed cases. Control strategies are widely used in order to reduce the incidence of canine disease and consequently the disease in humans. However, despite efforts to control the disease, the incidence of human and canine VL is still high. This fact can be attributed to the low sensitivity of the diagnostic tests used in epidemiological surveys and the length of time between the dogs' diagnosis and treatment. Another critical factor for the permanence of the disease is the fact that in areas endemic for VL, more than 80% of infected dogs are asymptomatic and may not be properly diagnosed by serological tests, due to the low number of antibodies produced by the immune system of the dog host or because it is the initial stage of the disease. Although the clinical disease is not observed in these animals, they are carriers of *Leishmania* and, therefore, are a relevant source of the parasite reservoir. Therefore, serological tests capable of diagnosing early infections in positive dogs, especially asymptomatic ones, remain an important issue for VL control.

Serological methods are important tools in the diagnosis of VL, since this disease is characterized by a large production of host-specific antibodies and the stimulation of polyclonal B cells. Serological techniques, such as the enzyme-linked immunosorbent assay (ELISA) and the rapid immunochromatographic test (ICT) are relatively simple, easy to perform, have quick and low-cost results, and can be performed manually or automated. The ICT still has the advantage of being independent of technology and qualified labor, which makes this test an excellent tool for mass screening, mainly in the field. These techniques allow the use of different types of antigens, such as crude or soluble extract of *Leishmania* ssp. or recombinant proteins. Recombinant molecules with varying sensitivity and specificity have emerged as an alternative to improve the quality of this type of diagnosis. Recombinant molecules are an integral part of the currently available immunological tests for the disease and could form the basis for new diagnostic tools. In any case, it is necessary to find the most sensitive targets to the point of reducing the existing variability of the humoral response of patients or dogs. More molecules that are specific are also needed, that is, molecules composed only of portions that are relevant in the antigen-antibody interaction exclusive for leishmaniasis. This will lead to the development of better and more accurate tests.

Over the years, several recombinant proteins from *L. infantum* and *L. donovani* have been characterized and evaluated, proving to be useful in the discrimination of VL. In this thesis, a new recombinant and synthetic antigen called

KDDR-plus was characterized, which is predominantly based on the repetitive portion found in the kinesin protein of *L. infantum*, being composed of 15.3 repetitions in a sequence of 39 amino acids. Initially, rKDDR-plus was used as an antigenic target in the serological diagnosis of VL, showing promising results in ELISA assays, both in human diagnosis and in the recognition of canine sera. In the serological ELISA assays, using human sera, the sensitivity and specificity of the test was 98% and 100%, respectively, and when evaluating canine sera, the sensitivity was 97% and the specificity 98%, indicating its potential for use in diagnosis VL. Subsequently, the performance of a rapid test was evaluated in a point of care format based on the rKDDR-plus antigen. The rapid lateral flow test (Lateral Flow Test) called ICT/KDDR-plus was evaluated against human and dog sera infected with *Leishmania*. Among the characteristics of the evaluated test, it is noteworthy that it has a low manufacturing cost, easy production on a large scale, relatively long shelf life without the need for refrigeration, the low volume of sample required for execution and obtaining results in a few minutes.

Another promising recombinant protein, characterized and tested in this study, is called Dyn-1, which stood out for its excellent ability to identify dogs with asymptomatic infections. rDyn-1 is a GTP (guanosine triphosphate) binding protein from *L. infantum* (LinJ.29.2310) selected from the ImmunonomeBase database due to its similarity with proteins associated with host defense processes. This protein was able to recognize sera from asymptomatic dogs, as well as from symptomatic ones. In serological ELISA assays, the rDyn-1 protein was able to recognize 100% of dogs considered asymptomatic.

Detailed screenings of the complete sequences of the proteins were carried out to expose the peptides responsible for the most relevant characteristics in both proteins. The peptides present in the rDyn-1 protein responsible for the ability to identify symptomatic, as well as asymptomatic ones, and the peptides present in the rKDDR-plus protein responsible for the ability to discriminate dogs that have *L. infantum* infection without showing cross-reaction with other organisms, were identified. In order to identify potential immunologically competent targets specific selection criteria were defined to identify targets with the highest score. For this purpose, a selection of epitopes of the recombinant Dyn-1 and KDDR-plus proteins was carried out, through screening in immunoblotting assays with pools of canine sera infected with *L. infantum* (asymptomatic and symptomatic) and non-infected (healthy dogs) and dogs infected with *T. cruzi*, *Babesia* sp. or *Ehrlichia* sp. for the selection of potential candidates for VL diagnosis. Thus identifying a peptide mixture composed of amino acid sequences of two recombinant proteins (Dyn-1 and KDDR-plus) with potential application in the diagnosis of CanL.

However the use of recombinant proteins has improved the sensitivity and specificity of the diagnosis. However, a new generation of antigens has been gaining prominence for further improving the diagnostic accuracy of immunological methods. Greater sensitivity and specificity can be achieved using multiepitope antigens, that is, multiple grouped peptide sequences composed of several fragments of more exposed epitopes unique to the molecule of interest. A judicious selection of the central antigenic parts of the native proteins generating short peptide fragments could refine and reduce the variability of the humoral response of patients and dogs. These

molecules still have the advantage of being simpler and cheaper to produce than whole recombinant proteins. In this perspective, the search and improvement of new targets for the diagnosis of VL is the way to quickly and effectively control the disease. Because, tests that present satisfactory sensitivity minimize the number of false negative cases, and tests with high specificity, avoid false positive results. The correct diagnosis allows the immediate initiation of treatment, when indicated, and helps in the epidemiological surveillance of dogs on a larger scale, helping to make clinical and epidemiological decisions, thus promoting better disease control actions. Therefore, from the results obtained so far in this study, it is expected to actually contribute to the control of VL, through the development of more efficient tests, and the encouragement of the production of new technologies not only for VL, but also for other infectious diseases.

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8. Impact paragraph

This thesis focused on one of the most important infectious disease challenges in Latin America, visceral leishmaniasis (VL). In addition to the existing socioeconomic challenges behind this disease, there are also scientific challenges to the successful control of leishmaniasis. The zoological character of this disease, in which dogs are seen as villains, means that control measures are not restricted to humans only. Therefore, this thesis focuses on improving human and especially canine diagnosis of the disease, which is one of the main disease control measures.

In this thesis, it is demonstrated that it is possible to improve the performance of human and canine serological diagnosis with the discovery of a new antigen that substantially increased the specificity both in ELISA assays and in immunochromatographic tests. Proudly, as an impact, we can say that the study presented in Chapter 2 of this thesis brought excellent results, resulting in a positive social impact by overcoming academic barriers, reaching the population that actually needs this service. This was demonstrated by the fact that in a short period since its discovery rKDDR-plus was patented (Figure 23), the patent was licensed and the antigen is currently being widely used in immunochromatographic tests for the diagnosis of the disease in Belo Horizonte, Minas Gerais/Brazil, showing good commercial but also societal impact (Figure 24). The discovery of rKDDR-plus was considered a milestone for this laboratory, being a reference for the search for more molecules with the same potential for the diagnosis of other infectious diseases.

Another positive impact generated by the thesis was the introduction of a new protein family to study to improve the diagnosis of CanL stabbed mainly in the identification of asymptomatic infections by *Leishmania*, which still lacks more targeted research. The rDyn-1 protein was able to identify 100% of cases of dogs without clinical signs of leishmaniasis. The population of dogs that goes unnoticed by most of the antigens used in the diagnosis of the disease has a great influence on the spread of the disease. It is also expected that rDyn1 will be introduced to epidemiological studies of VL, allowing the determination of the real prevalence and incidence rate, mainly in endemic regions. This could further improve understanding the disease and, in turn, controlling it with appropriate containment measures.

Following the current trend of using recombinant antigens as sensitizers, we show here the benefits generated by the systematic use of bioinformatics tools together with immunological techniques for faster and more efficient identification of these antigens. The use of bioinformatics tools was a facilitator and accelerator in this work, saving resources and mainly time, as it provides multiple filters before performing *in vitro* tests. We judge as another repercussion the importance of more meticulous studies directed to the identification of more specific portions of whole proteins responsible for peculiar characteristics of previous interest. This more targeted study made it possible to identify peptide portions with specific characteristics that, when mixed, proved capable of achieving performance equal to or greater than their individual use.

Thus, several premises were pioneered here, such as the use of a more specific antigen, the inclusion of a new family in diagnostic studies of asymptomatic dogs with leishmaniasis, and the screening of smaller portions of proteins with more

defined characteristics than whole proteins. The present work can contribute to the development of more efficient methodologies for the diagnosis of canine and human visceral leishmaniasis, which ultimately might positively influence disease control in Brazil. As a perspective, the development of new diagnostic platforms will allow control not only for VL but also serve as an example for creating tools for the diagnosis and containment of other neglected diseases.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Número do Processo: BR 13 2017 028144 1

Dados do Depositante (71)

Depositante 1 de 2

Nome ou Razão Social: UNIVERSIDADE FEDERAL DE MINAS GERAIS
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 17217985000104
Nacionalidade: Brasileira
Qualificação Jurídica: Instituição de Ensino e Pesquisa
Endereço: Av. Antônio Carlos, 6627 - Unidade Administrativa II - 2º andar- sala 2011
Cidade: Belo Horizonte
Estado: MG
CEP: 31270-901
País: Brasil
Telefone: (31) 34094774
Fax:
Email: patentes@ctit.ufmg.br

PETICIONAMENTO ELETRÔNICO

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Depositante 2 de 2

Nome ou Razão Social: FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE MINAS GERAIS
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 21949888000183
Nacionalidade: Brasileira
Qualificação Jurídica: Pessoa Jurídica
Endereço: Av. José Cândido da Silveira, 1500, Horto
Cidade: Belo Horizonte
Estado: MG
CEP: 31035-536
País: BRASIL
Telefone: (31) 340 94774
Fax:
Email: patentes@ctit.ufmg.br

Dados do Pedido

Natureza Patente: 13 - Certificado de Adição (C)
Pedido Original: BR1020120324997
Título da Invenção ou Modelo de Utilidade (54): "PROTEÍNA RECOMBINANTE, KIT PARA DIAGNÓSTICO DA LEISHMANIOSE VISCERAL HUMANA E CANINA, COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSE E USOS"
Resumo: A presente tecnologia descreve a proteína recombinante rKDDR-plus e seu uso para o diagnóstico da leishmaniose visceral humana e canina (LVH e LVC), com alta precisão e acurácia na detecção de humanos e/ou cães infectados por Leishmania, auxiliando no controle da transmissão e no tratamento da leishmaniose visceral. A proteína rKDDR-plus apresenta um alto grau de repetição do motivo repetitivo de 39 aminoácidos derivado da Kinesina, o que permite um diagnóstico da leishmaniose visceral humana e canina com maior sensibilidade e especificidade, além de utilizar menor quantidade de proteína nos kits diagnósticos, em relação às outras proteínas disponíveis no mercado. A rKDDR-plus pode também ser utilizada em uma composição vacinal contra leishmaniose.
Figura a publicar: 5

PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 26/12/2017 às 15:37, Petição 870170101905

ção 870170101905, de 26/12/2017, pág. 2/39

Figure 23 - Patent deposit. Invention addition deposit and entry into the national phase of the PCT.

DECLARATION

To whom it may concern,

SAFETEST DIAGNOSTICOS LTDA, a Brazilian biotechnology company focused on development of rapid tests, declares that it has the exclusive license for use of KDDR (and its derivatives products as KDDR-Plus) antigen for serological detection of Visceral Leishmaniasis. In Brazil, the immunochromatographic test based on the KDDR-Plus antigens is currently one of the main products used for detection of this tropical disease in dogs.

Belo Horizonte, Brazil. March 10th, 2023.

Felipe Augusto Lins Peixoto
SAFETEST DIAGNOSTICOS LTDA

Figure 24 - Declaration Safe Test Diagnósticos. Statement the company that owns the license and marketing rights for products derived from rKDDR-plus

9. List of publications

9.1. Patent application

Siqueira WF, Fujiwara RT**,** Bartolomeu DC, Cardoso MS, Bueno LL, Dhom Lemos, LC, Cunha JLR, Mendes AO, Lobo FP. Proteína recombinante, kit para diagnóstico da leishmaniose visceral humana e canina, composição vacinal contra leishmaniose e usos. 2017, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1320170281441, título: "Proteína recombinante, kit para diagnóstico da leishmaniose visceral humana e canina, composição vacinal contra leishmaniose e usos", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 26/12/2017; Licenciamento: 27/12/2017. Instituições) financiadora (s): CAPES, FAPEMIG, CNPq.

9.2. Published book chapters

Siqueira WF, Cardoso MS, Oliveira ALG, Bartolomeu DC, Bueno LL, Fujiwara RT. Leishmaniose visceral: contribuições históricas e avanços no diagnóstico para o controle da doença. In: Atualidades em Medicina Tropical no Brasil: Protozoários.1 ed.: StrictoSensu Editora, 2020, p. 288-305 (presented as chapter in this thesis).

Oliveira ALG, Cardoso MS, **Siqueira WF**, Bartolomeu DC, Bueno LL, Fujiwara RT. Desafios e avanços nos diagnósticos sorológico e molecular da hanseníase. In: Atualidades em Medicina Tropical no Brasil: Microbiologia.1 ed.: Stricto Sensu Editora, 2020, p. 64-80.

9.3. Publications presented as chapters in this thesis

Siqueira WF, Cardoso MS, Fraga VG, Ottino J, Ribeiro VM, Gondim CN, Barçante JMP, Gomes ACA, Galdino AS, Eersels K, Grinsven BV, Bartholomeu DC, Bueno LL, Cleij T, Fujiwara RT. Immunogenic mapping of rDyn-1 and rKDDR-plus proteins and selection of oligopeptides by immunoblotting for the diagnosis *of Leishmania infantum*-infected dogs. PLoS Neglected Tropical Diseases, v. 17, p. e0011535, 2023.

Siqueira WF, Cardoso MS, Clímaco MC, Silva ALT, Heidt B, Eersels K, van Grinsven B, Bartholomeu DC, Bueno LL, Cleij T, Fujiwara RT. Serodiagnosis of leishmaniasis in asymptomatic and symptomatic dogs by use of the recombinant dynamin-1-like protein from *Leishmania infantum*: A preliminary study. Acta Trop. 2023 Mar;239:106827. doi: 10.1016/j.actatropica.2023.106827. Epub 2023 Jan 5. PMID: 36610530.

Siqueira WF, Viana AG, Reis Cunha JL, Rosa LM, Bueno LL, Bartholomeu DC, Cardoso MS, Fujiwara RT. The increased presence of repetitive motifs in the KDDR-plus recombinant protein, a kinesin-derived antigen from *Leishmania infantum*, improves the diagnostic performance of serological tests for human and canine visceral leishmaniasis. PLoS Negl Trop Dis. 2021 Sep 17;15(9):e0009759. doi: 10.1371/journal.pntd.0009759. PMID: 34534217; PMCID: PMC8480608.

9.4. Publications as co-author

Santos TTO, Machado AS, Ramos FF, Oliveira-da-Silva JA, Lage DP, Tavares GSV, Mendonça DVC, Cardoso MS, **Siqueira WF**, Martins VT, Ludolf F, Reis TAR, Carvalho LM, Freitas CS, Bandeira RS, Silva AM, Oliveira JS, Moreira RLF, Fujiwara RT, Roatt BM, Chávez-Fumagalli MA, Humbert MV, Teixeira AL, Coelho EAF. *Leishmania* eukaryotic elongation Factor-1 beta protein is immunogenic and induces parasitological protection in mice against *Leishmania infantum* infection. Microb Pathog. 2021 Feb;151:104745. doi: 10.1016/j.micpath.2021.104745. Epub 2021 Jan 21. PMID: 33485994.

Vale DL, Lage DP, Machado AS, Freitas CS, de Oliveira D, Galvani NC, Fernandes BB, Luiz GP, Oliveira JS, Oliveira-da-Silva JA, Ramos FF, Santos TTO, **Siqueira WF**, Alves LA, Chávez-Fumagalli MA, de Magalhães-Soares DF, Silveira JAG, Bueno LL, Fujiwara RT, Machado-de-Ávila RA, Martins VT, Coelho EAF. Serodiagnosis of canine leishmaniasis using a novel recombinant chimeric protein constructed with distinct B-cell epitopes from antigenic *Leishmania infantum* proteins. Vet Parasitol. 2021 Aug;296:109513. doi: 10.1016/j.vetpar.2021.109513. Epub 2021 Jul 1. PMID: 34225189.

Heidt B, **Siqueira WF**, Eersels K, Diliën H, van Grinsven B, Fujiwara RT, Cleij TJ. Point of Care Diagnostics in Resource-Limited Settings: A Review of the Present and Future of PoC in Its Most Needed Environment. Biosensors (Basel). 2020 Sep 24;10(10):133. doi: 10.3390/bios10100133. PMID: 32987809; PMCID: PMC7598644.

Dhom-Lemos L, Viana AG, Cunha JLR, Cardoso MS, Mendes TAO, Pinheiro GRG, **Siqueira WF**, Lobo FP, Teles LF, Bueno LL, Guimarães-Carvalho SF, Bartholomeu DC, Fujiwara RT. *Leishmania infantum* recombinant kinesin degenerated derived repeat (rKDDR): A novel potential antigen for serodiagnosis of visceral leishmaniasis. PLoS One. 2019 Jan 31;14(1):e0211719. doi: 10.1371/journal.pone.0211719. PMID: 30703138; PMCID: PMC6355020.

Abeijon C, Alves F, Monnerat S, Wasunna M, Mbui J, Viana AG, Bueno LL, **Siqueira WF**, Carvalho SG, Agrawal N, Fujiwara R, Sundar S, Campos-Neto A. Development of a Multiplexed Assay for Detection of *Leishmania donovani* and *Leishmania infantum* Protein Biomarkers in Urine Samples of Patients with Visceral Leishmaniasis. J Clin Microbiol. 2019 Apr 26;57(5):e02076-18. doi: 10.1128/JCM.02076-18. PMID: 30787142; PMCID: PMC6497999.

Santos TTO, Cardoso MS, Machado AS, **Siqueira WF**, Ramos FF, Oliveira-da-Silva JA, Tavares GSV, Lage DP, Costa LE, de Freitas CS, Martins VT, Bandeira RS, Chávez-Fumagalli MA, Lyon S, Moreira RLF, de Magalhães-Soares DF, Silveira JAG, Tupinambás U, Caligiorne RB, Chaves AT, Rocha MOC, Fujiwara RT, Coelho EAF. Recombinant *Leishmania* eukaryotic elongation factor-1 beta protein: A potential diagnostic antigen to detect tegumentary and visceral leishmaniasis in dogs and humans. Microb Pathog. 2019 Dec;137:103783. doi: 10.1016/j.micpath.2019.103783. Epub 2019 Oct 7. PMID: 31600536.

10. Curriculum vitae

Williane Fernanda Siqueira was born on April 11 of 1991 in the city of Nova Lima, Brazil. She obtained a Bachelor of Science degree in Biological Sciences with emphasis on biotechnology at the Centro Universitário UNA, Belo Horizonte, Brazil in 2013, followed by a master's degree in parasitology at the Federal University of Minas Gerais (UFMG) in 2018. She has 10 years of experience in infectious disease research. She has been working in diagnostic laboratories for infectious and parasitic diseases in the research and identification of molecular targets to be used in different diagnostic platforms. In August 2018, she began to obtain her Ph.D. at the department of Infectology and Tropical Medicine at UFMG. Then started her double Ph.D. at Maastricht University based on a cooperation agreement between the UFMG and the Sensor Engineering Department of the Faculty of Science and Engineering at Maastricht University. Ph.D. students in a dual degree program, receive two Ph.D. diplomas, one from each university. Her research interests and expertise are mainly focused on the development of diagnostic tests for the control of neglected tropical diseases. Here, her research focused on the identification of molecules, *in silico*, and validation, *in vitro*, such as rapid tests and a Point of Care (POC) diagnostic device for infectious diseases. Her scientific output includes one patent, two book chapters, nine peer-reviewed articles published. Three of these peer-reviewed articles are the results of the partnership between UFMG and the Maastricht University. Section 9.2.1 presents the list of all her scientific production.

Acknowledgements

I thank God for bestowing the gift of life and for granting me physical and mental health to pursue His purposes. For making me understand that, it is not about my desires or me but about his designs and desires. I am infinitely grateful for putting in my path people, mentioned here, who were the foundation for me to achieve his purposes. "The Lord knows exactly what's best for me."

First, I would like to thank my family for without their ongoing and unwavering support my complete career and going on this journey would not have been possible.

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To my godmother, Maria de Fátima (Xá), for making I awaken the taste for studies since I was a little girl by the simple gesture of binding my books. For being such an intelligent and dedicated person. Thank you for your support, care and dedication.

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To my supervisor, Ricardo Toshio Fujiwara, I thank you for welcoming me into your laboratory and for entrusting me with the responsibility of an extremely important project, in addition to providing the best working conditions so that I could complete this research successfully. For being this brilliant teacher, scientist and researcher who has the gift of words and the gift of encouraging the people around him. Thank you for helping me to become a real scientist.

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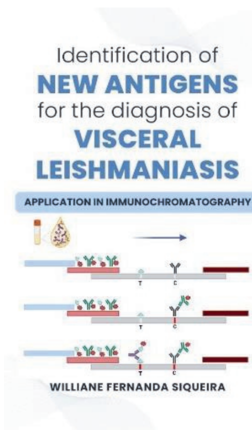

This thesis focused on one of the parasitic infectious disease challenges in Latin America and Europe, visceral leishmaniasis (VL). In addition to the existing socioeconomic challenges, there are also scientific challenges to the successful control of leishmaniasis. The zoological character of this disease, in which dogs are seen as villains, means that control measures are not restricted to humans only. Therefore, this thesis focuses on improving human and especially canine diagnosis of the disease, which is one of the main disease control measures. In this thesis, it is demonstrated that it is possible to improve the performance of human and canine serological diagnosis with the discovery of new antigens that increase the specificity of the serological tests.

WILLIANE FERNANDA SIQUEIRA

obtained a bachelor's degree in Science degree in Biological Sciences with emphasis on biotechnology at the Centro Universitário UNA, Belo Horizonte, Brazil, followed by a master's degree in Parasitology at the Federal University of Minas Gerais (UFMG), Brazil. Acting since 2016 in the identification of antigenic molecules for research and development of diagnostic tests for infectious diseases. She has extensive knowledge of heterologous expression of recombinant proteins, molecular biology, and serological techniques. With experience in the development of rapid tests and diagnostic devices such as Point of Care (POC) and in vitro diagnostic (IVD) devices. In August 2018 she began to obtain her double doctoral at the Sensor Engineering Department of the Faculty of Science and Engineering at Maastricht University and at the Faculty of Medicine at the UFMG in Medicine and Life Science. Here, her research focused on the identification and selection of new molecules for application in serological tests and biosensors.