UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
KATEDRA BIOLOGICKÝCH A LÉKAŘSKÝCH VĚD

Optimalizace diagnostiky leišmaniózy u psů

Diplomová práce

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Hradec Králové 2015 Barbora Danková

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DEPARTMENT OF BIOLOGICAL AND MEDICAL SCIENCES

Optimalization of *Leishmania* antigen formulation for the diagnosis of canine leishmaniasis

Diploma thesis

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"We have not disclosed in detail certain aspects of the proposed work as there is a possibility of seeking patent protection and we do not wish to create any potential prior art which may prevent a patent."

Abstrakt

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Název práce: Optimalizace diagnostiky leišmaniózy u psů

Leišmanióza je parazitální onemocnění způsobené prvokem z rodu Leishmania, šířící se pomocí přenašeče. Vyskytuje se v 98 zemích po celém světě.

Pro laboratorní diagnózu leišmaniózy jsou nezbytné spolehlivé a přesné testy, protože se může vyskytnout široké spektrum klinických znaků, symptomů a vysoká míra asymptomatických infekcí. Sérologické stanovení leišmaniózy u psů, zejména metoda enzyme-linked immunosorbent assay (ELISA), se ukázala být užitečnou.

Údaje získané z ELISA testu pro detekci protilátek proti vybraným rekombinantním proteinům A, B, C, D, E a F byly vyhodnoceny z infekčních sér, které byly získány v Portugalsku a Brazílii. Tato práce prezentuje nejlepší formulaci rekombinantních proteinů pro sérologické stanovení diagnózy. Zjistili jsme, že antigen C ukázal vysoký stupeň senzitivity a specificity v rozpoznání pozitivních a asymptomatických sér infikovaných leišmaniózou.

Klíčová slova: leišmanióza u psů, rezervoár, diagnóza, ELISA.

Abstract

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Title of diploma thesis: Optimalization of *Leishmania* antigen formulations for the diagnosis of canine leishmaniasis

Leishmaniasis is a vector-borne disease caused by a protozoan parasite of the genus *Leishmania* and is prevalent in 98 countries worldwide.

Reliable and accurate tests are necessary for laboratory diagnosis of *Leishmania* infection because of the wide spectrum of clinical characteristics and symptoms and high rate of asymptomatic infections that may occure. Serological diagnosis of canine leishmaniasis (CanL), especially enzyme-linked immunosorbent assay (ELISA), proved to be usefull tool.

Data obtained from ELISA assay for the detection of antibodies against selected recombinant A, B, C, D, E and F proteins were evaluated in infected canine sera from Portugal and Brazil. This work presents the best formulation of recombinant proteins for serological diagnosis. We found that antigen C showed high level of sensitivity and specificity in recognition of positive and asymptomatic sera infected with CanL.

Key words: canine leishmaniasis, reservoir, diagnosis, ELISA.

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1. **LIST OF ABBREVIATIONS**

AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine Triphosphate
Br	Brazil
CanL	Canine Leishmaniasis
CL	Cutaneous Leishmaniasis
CVL	Canine Visceral Leishmaniasis
DAT	Direct Agglutination Test
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAST	Fast Agglutination Screening Test
FC	Flow Cytometry
FC-AFPA-IgG	Anti-fixed *Leishmania chagasi* Promastigotes IgG Antibodies Detected by Flow Cytometry
FC-AFPA-IgG1	Anti-Fixed *Leishmania chagasi* Promastigotes IgG1 Antibodies Detected by Flow Cytometry
FC-AFPA-IgG2	Anti-Fixed *Leishmania chagasi* Promastigotes IgG2 Antibodies Detected by Flow Cytometry
GTP	Guanosine Triphosphate
ICT	Immunochromatographic Test
IFAT	Indirect Fluorescent Antibody Test
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G subclass 1
IgG2	Immunoglobulin G subclass 2
IgM	Immunoglubulin M
IL-10	Interleukin 10
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5

IL-6	Interleukin 6
kDNA	kinetoplast DNA
LAM	*Leishmania* Antigen Mixture (combination of *Lic*TXNPx 1µg/ml + rK39 4µg/ml)
*Lic*TXNPx	*Leishmania infantum* cytosolic Tryparedoxin Peroxidase
*Lim*TXNPx	*Leishmania infantum* mitochondrial Tryparedoxin Peroxidase
MCL	Mucocutaneous Leishmaniasis
NO	Nitric Oxide
PCR	Polymerase Chain Reaction
PKDL	Post-Kala azar Dermal Leishmaniasis
Pt	Portugal
QDs	Quantum Dots
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
rA2	recombinant A2 antigen
rK26	recombinant K26 antigen
rK28	recombinant K28 antigen
rK29	recombinant K29 antigen
rK39	recombinant K39 antigen
rK9	recombinant K9 antigen
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
SALA	Soluble Amastigote *Leishmania* Antigens
SPLA	Soluble Promastigote *Leishmania* Antigens
TGFβ	Transforming Growth Factor beta
Th1	T helper type 1 cells
Th2	T helper type 2 cells
TNF-α	Tumor Necrosis Factor alpha
T-reg	Regulatory T cell
VL	Visceral Leishmaniasis
VL-HIV coinfection	Visceral Leishmaniasis - Human Immunodeficiency Virus coinfection

2. INTRODUCTION

Leishmaniasis is a worldwide parasitic disease caused by obligate intracellular protozoan of the genus *Leishmania* (Trypanosomatidae). Three main clinical forms of the disease are described: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL, also known as kala-azar). The outcome of infection is determined by involved *Leishmania* species and immune susceptibility of the host. Leshmaniasis is transmitted by the bite of infected female sandfly [1,2].

Visceral leishmaniasis, caused by *L. infantum*, is a severe zoonosis with important animal reservoir in domestic dogs and it is potentially fatal for both, humans and dogs if not treated [2,3].

CanL spreads quickly in endemic areas and also in non-endemic countries due to increased human travelling and climate changes. The main problem is a high rate of subclinical infections because not all infected dogs develop clinical signs of the disease, and they can stay asymptomatic [4,5].

Accurate diagnosis remains a problem because a wide range of clinical signs can occur. Early and reliable diagnosis is required for the detection of symptomatic and asymptomatic infections. An ideal test should be sensitive, specific, easy to perform and non-expensive [3,6]. Laboratory diagnosis can be performed by using direct and indirect methods. The most powerful techniques include determination of *Leishmania* amastigotes in tissues, detection of the parasite DNA and detection of specific anti-*leishmania* antibodies using immunological tests, like direct agglutination test (DAT), indirect fluorescent antibody test (IFAT) and ELISA [3,7].

3. THEORETICAL PART

3.1. Epidemiology

Leishmaniasis is a disease caused by protozoan intracellular parasite of the genus *Leishmania*. This disease is endemic in 98 countries worldwide, in many tropical and subtropical areas including Mediterranean basis, North Africa, Central and South America, the Middle East of the Indian subcontinent (Fig.1). Leishmaniasis is the most important vector-borne disease after malaria and sleeping sickness [8-10].

The annual incidence of the disease is about 2 million (1.5 million are cutaneous, and 500 000 are visceral form). The mortality is more than 50 000 individuals per year. More than 350 million people were noticed to be at risk of infection [5,11]. The vast majority cases of leishmaniasis (>90%) were observed in Brazil, India, Bangladesh, Sudan, Ethiopia and Nepal. Leishmaniasis usually afflicts poorer communities, generally in faraway areas [11].

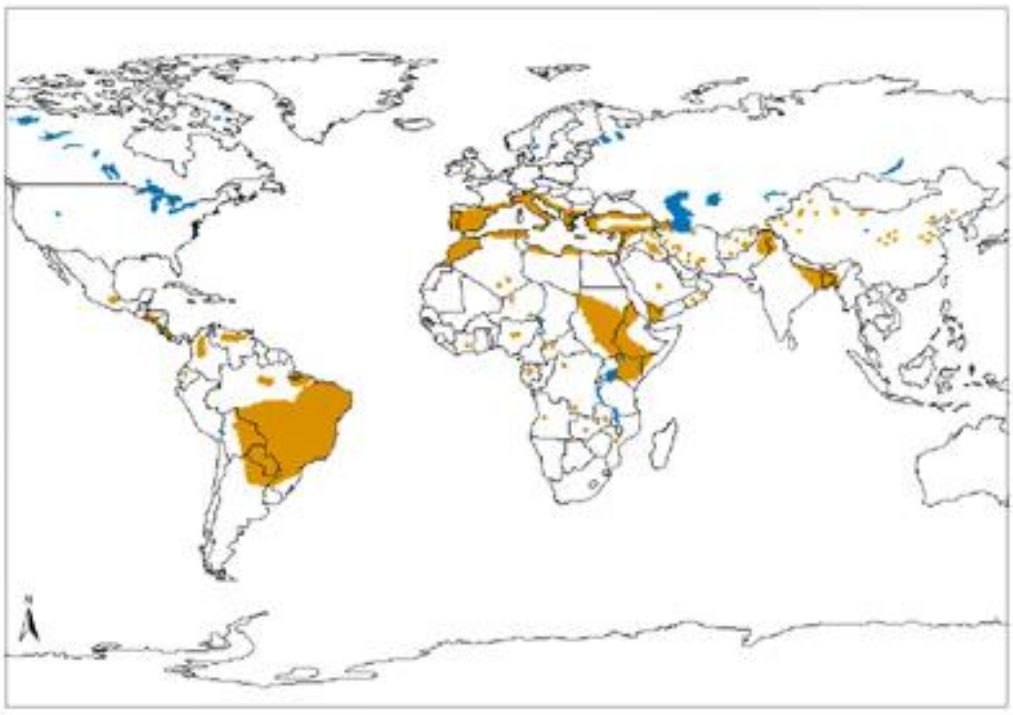


Fig. 1 Distribution of visceral leishmaniasis (VL) worldwide, 2010. Adapted from http://www.who.int/leishmaniasis/leishmaniasis_maps/en/ (11.1.2015)

The major factors responsible for increasing incidence of the disease are socioeconomic conditions, population mobility, environmental and climate changes, malnutrition and anti-leishmanial drug resistence. Almost all human cases occur because of sandfly bites, contaminated blood and intravenous drug users due to sharing needles [2].

Increased incidence of human leishmaniasis has been recorded in 1990s, mostly under the influence of human immunodeficiency virus. This coinfection is considered an urgent disease in the area of southern Europe [8].

Over 20 species of *Leishmania* are able to infect humans, animals and sandflies, the vector of the disease. Prevalence of organism depends on geographical distribution. *Leishmania* species are classified into two groups: Old World (Africa, Mediterranean area, Middle East, Far East) – *L. donovani, L. infantum, L. tropica, L. major, L. aethiopica* and New World (Central, South and North America) – *L. infantum (*syn. *L. chagasi), L. mexicana, L. amazonensis, L. panamensis, L. braziliensis, L. guyanensis, L. peruviana* and *L. venezuelensis* [2,9,12].

3.2. Life cycle and transmission

Leishmania spp. are transmitted to humans and animals by the sandfly's bite. There are about 30 various species of sandfly. In New World *Lutzomyia* is the most common genus and in The Old World is *Phlebotomus* [1,8].

The sandfly usually feeds from dusk to dawn. Its highest activity is during the warm months, between 15 and 28°C, with high relative humidity and lack of rains. Only female sandflies are able to transmit the disease [5,11].

The life cycle of *Leishmania* spp. has two main different phases. In one of the phases, a promastigote is found in the anterior segment of the gut of the sandfly vector. Promastigote is extracellular and motile, with an elonged shape with flagellum. In the other phase of the cycle an intracellular amastigote is found in the mammalian host, and is characterized by ovoid shape without flagellum [5,11] (Fig.2).

There is a connection between *Leishmania spp*. and sandfly species. In the gut of insect are localised ligands and enzymes with specific activity that permit only certain *Leishmania* species to stay attached to the gut wall [5].

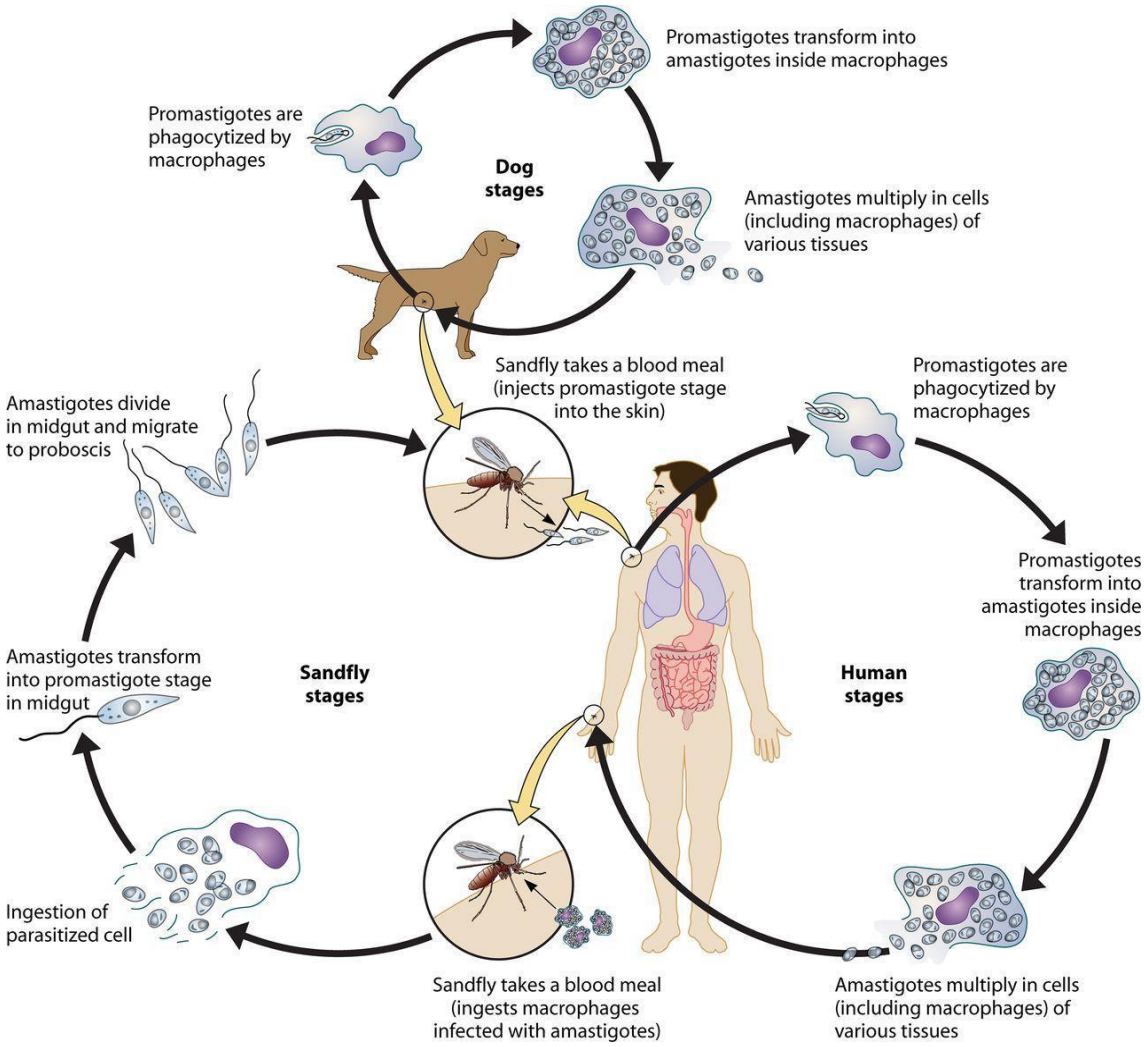


Fig. 1 Leishmania life cycle. Copy from: http://galleryhip.com/babesia-microti-life-cycle.html (2.10.2014)

The sandfly transmits the disease by injecting promastigotes into the mammal skin during the feeding. They are quickly surrounded and phagocytized by dendric cells and macrophages in the dermis. Inside the body, promastigotes are transformed into amastigotes. They multiply through simple division in phagolysosomes and diffuse via vascular and lymphatic systems. The parasites can infect other macrophages and monocytes in reticulo-endothelial system, and spread to the kidneys, liver, reproductive organs, urinary bladder, skin, respiratory and digestive system. During the feeding on an infected animal (host), sandflies ingest macrophages with amastigotes. In the sandfly, the amastigotes develop into promastigotes that will be injected into the mammalian host during the next feed [9,11,13,14].

The transmission of leishmaniasis can be divided into two main types: zoonotic and anthroponotic. Zoonotic form occurs in the Mediterranean Basin, Brazil and some parts of Africa. Transmission from animal to vector and to human is typical for this form and it is caused by *L. infantum*. The other form, anthroponotic, occurs from human to vector and to human. In this type of transmission pattern the absence of a reservoir is typical. Parasites are transmitted only between human hosts. It is more common in the Indian subcontinent and it is caused by *L. donovani*. This transmission can happen in intravenous drug users [6,9-11,15].

3.3. Clinical presentation

Leishmaniasis is classified into three main different forms: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL). The character of this disease depends on the type of *Leishmania* species and on the immune response of the host [2,9] (Fig.3).

3.3.1. Cutaneous leishmaniasis

This type of leishmaniasis is the most common form of the disease and it may advance to other forms. CL usually starts as skin nodules in sandfly bites region, which can enlarge and ulcerate. These lesions are typically localized in the exposed areas of the body (e.g. the face, legs and forearms). Histologically, CL is described by a monocytic and lymphoid infiltrate with granuloma formation in the dermis [1,2,9].

The incubation period is from two weeks to few months after the first bite. In most cases, lesions heal spontaneously, in the period from 3 to 18 months because of developed aquired immunity of the host [9,15].

3.3.2. Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis is characterized by chronic and local destruction of the mucosa. MCL involves the nose, oral cavity, oropharynx, nasopharynx and eyelids. Patients suffer from nose bleeding, ulceration and perforation of the nasal septum. It can affect the respiratory function and cause problems with eating. Secondary infection and malnutrition are the main reasons responsible for mortality of MCL [1,9].

The incubation period is from 1 to 3 months and in contrast with cutaneous form, these ulcers are not self-healing. They usually appear months or years after the initial episode of cutaneous leishmaniasis. When mucocutaneous leishmaniasis is not treated it can progress to extensive destruction of the lips, palate and cheeks [1,9,11].

3.3.3. Visceral leishmaniasis

Visceral leishmaniasis is a systemic disease caused by *Leishmania donovani* complex which contains three species*: L. infantum, L. chagasi* and *L. donovani*. This type of the disease is the most severe form. It is progressive and can be fatal if left untreated [11].

The incubation period from sandfly bite to presentation of the disease varies from 2 weeks to 18 months, but can be longer. Patients with VL present irregular fever, fatigue, weight loss, weakness and loss of appetite. These symptoms are caused because of long-term and systemic infection [11,16].

VL is characterized by spreading of parasites through the blood and reticulo-endothelial system and features include enlarged lymph nodes, hepathosplenomegaly, lymphadenopathy, anemia and pancytopenia [11]. The bone marrow suppression and hepatosplenomegaly are caused by parasitic proliferation inside the macrophages in the bone marrow, liver and spleen. When patients are not treated, pancytopenia and immunosuppression can evolve and they can be more susceptible to the secondary bacterial infections. Signs and symptoms of this coinfection (the diarrhoea, pneumonia or tuberculosis) may confuse the clinical picture of the disease [6,11,12].

Human VL infects mostly young children and people suffering from AIDS (acquired immune deficiency syndrome) [3]. The patients with VL-HIV coinfection can develop atypical presentation of the disease which involve the gastrointestinal tract (the stomach, duodenum or colon), lungs, tonsils and skin [6,12].

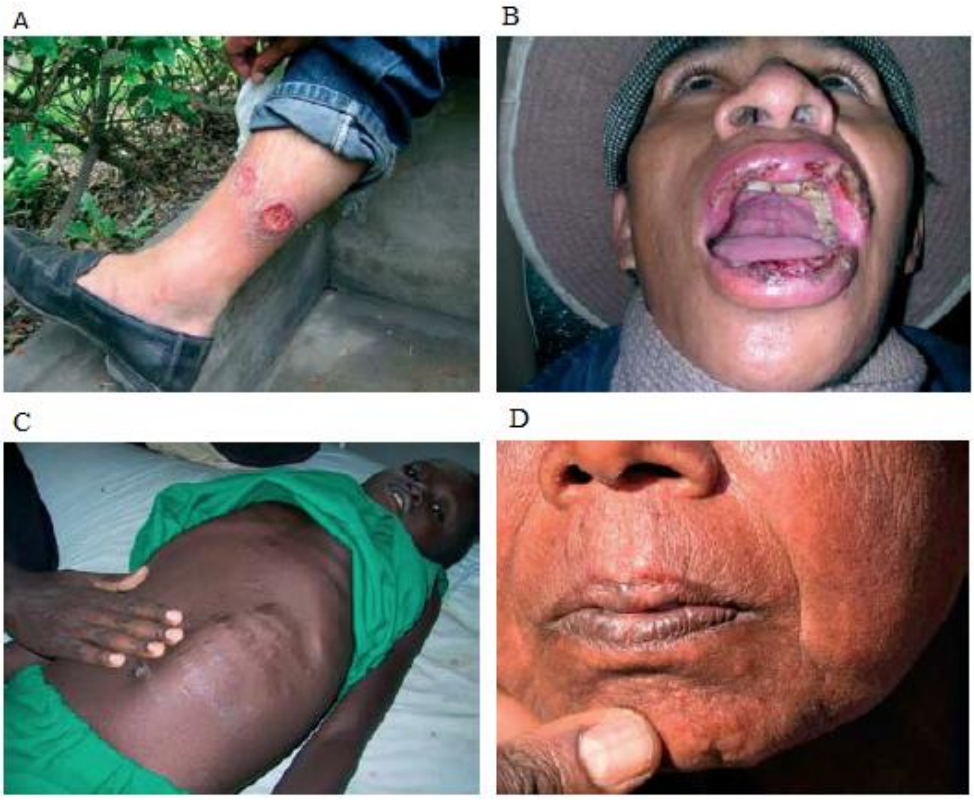


***Fig. 3 Clinical presentation of leishmaniasis*.** A) CL B) MCL C) VL D) PKDL (post-kala azar dermal leishmaniasis**)**, adapted from [11]

3.3.4. Post-kala azar dermal leishmaniasis

Post-kala azar dermal leishmaniasis (PKDL) is a complication of visceral leishmaniasis. This form is often observed after the treatment in African countries, Sudan and India, in patients infected by *L. donovani* [16].

In PKDL, patients can be without symptoms for months to years and after that they can develop signs and symptoms [16]. PKDL is characterized by a macular, maculo-papular or nodular skin lesions localized everywhere on the body, but especially on the face [9,16]. These lesions contain many parasites and therefore this type of disease is very infectious. The period between treated VL and PKDL depends on the geographical area (e.g. in Sudan is the interval 0-6 months, in India 6 months to 3 years) [11].

3.4. Immune response

The immune response of leishmaniasis incude both, cellular and humoral immunity [4].

In the cellular immunity, the result of infection depends on mixed Th1/Th2 response. Activated Th1 (T helper type 1) cells secrete cytokines, such as interleukin 2 (IL-2), interleukin 3 (IL-3), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). These cytokines are able to induce activation of macrophages and synthesis of NO synthase [9,14]. Nitric oxide (NO), which is produced by macrophages, is responsible for intracellular killing of *Leishmania* parasites by apoptosis [4]. In leishmaniasis, the Th1 response and low levels of specific antibodies are associated with resistance [9,14].

Th2 (T helper type 2) cells produce interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 10 (IL-10) and transforming growth factor beta (TGFβ) which induce production of antibodies by B-cells and induce increasing plasma-cell activity. These cytokines and high levels of specific antibodies are associated with susceptibility to infection and can cause disease progression. IL-10 is also secreted by T-regulatory cells (T-reg) and is considered to be a downregulator of Th1 response [4,9] (Fig.4)

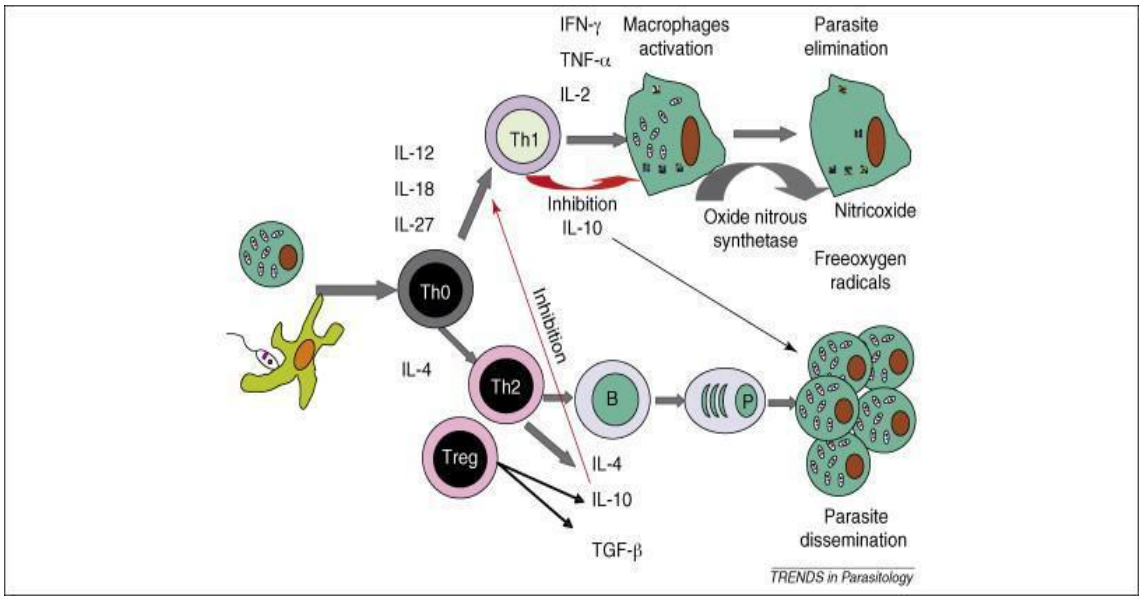


Fig. 4 Cell immune response, adapted from [4].

Humoral immune response is intense with high levels of *Leishmania*-specific antibodies. In infected dogs, different immunoglobulins are produced. The levels of anti-leishmanial antibodies are higher in symptomatic dogs. Lower levels were noticed in an initial and late phase of infection or in asymptomatic dogs [4,7].

Specific humoral immune response is presented by IgG (immunoglobulin G), IgG1 (immunoglobulin G subclass 1), IgG2 (immunoglobulin G subclass 2), IgM (immunoglobulin M), IgA (immunoglobulin A) and IgE (immunoglobulin E) [4,7]. Increased level of IgG1 was observed in asymptomatic carriers [17]. Higher level of IgG2 was found in symptomatic dogs. This immunoglobulin is considered to be a marker of disease [18]. IgM is produced after the initial phase of disease and formation of IgG. IgE was developed only in patients and animals which had clinical signs and symptoms [7].

3.5. Reservoir

Leishmaniasis is a zoonosis with very important animal reservoirs like canine and rodents [2,9].

Dogs, especially domestic, represent the main source of infection for human VL in the areas such as Mediterranean basin and South America [10]. Leishmaniasis in endemic areas is widespread and the prevalence of the disease has been estimated over 10% and can rise up to 35%. CanL can also appear in non-endemic areas and it is caused by transfer of dogs outside and inside of endemic areas [4]. The prevalence and incidence of CanL are very important epidemiological tools for infection control [10]. When conditions for transmission are optimal, the infection spreads extensively and rapidly among the dog population. The elderly dogs are more infected, males rather than females [4,14].

Not all infected dogs present signs and symptoms of disease, and so, some dogs are asymptomatic carriers. More than 50% of all infected dogs do not show any clinical characteristics but they are able to transmit the parasites to the vector. In asymptomatic dogs, low numbers of parasite are demonstrated in the blood, spleen, liver, lymph nodes and skin. Small percentage of dogs with CanL can spontaneously recover and eliminate the parasites [5,14].

The incubation period of CanL can be from three months to seven years [3]. Infected animals present a poor body condition, loss of weight, weakness, apathy, alteration of appetite, vomiting, diarrhoea, hypertermia, ocular lesions, generalized muscular atrophy, lameness, enlarged lymph nodes and spleen, anemia and nose bleeding. Dermal changes are the most common signs, including excessive skin scales, dermatitis, hyperkeratosis and depigmentation. These changes can be connected with immune response [17] (Fig.5). Opportunistic infection can also occur. The death is the outcome of hepatic or renal failure. The renal damage is caused by immune complex deposits [4,5,14].

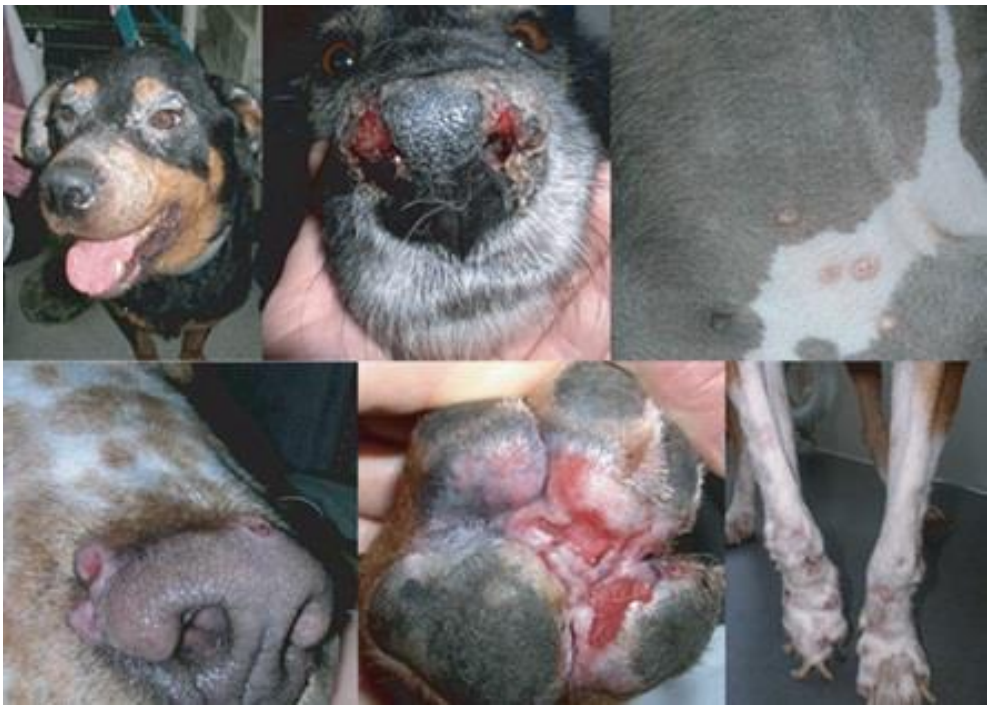


Fig. 5 Clinical manifestation of symptomatic canine leishmaniasis. From: http://www.lagosvet.com/diseases (31.12.2014)

For reducing the frequency of transmission of leishmaniasis from infected dogs to humans is important to control canine population and diagnose CanL as early as possible [19]. Accurate diagnostic tests are necessary for detection of infection in symptomatic and asymptomatic dogs [3].

There is an existence of non-sand fly transmission in dogs. Potential vectors also can be fleas and ticks. Venereal, transplacental, direct dog-to-dog transmission and transmission

due to infected canine blood during blood donation have been registered, but these cases play only small role in leishmania epidemiology [5].

As healthy seropositive dogs are able to infect sandflies, the use of deltamethrin or permethrin-impregnated dog collars, and spray preparations or topical insecticide repellents, reduce the risk of sandfly feeding [5,13]. Also, culling of seropositive dogs led to a decrease in the incidence of the disease, but this strategy is unacceptable in some countries for ethical and social reasons [10,20].

3.6. Treatment

There are various specific anti-leishmanial drugs. The treatment depends on the clinical form of the disease [12]. The main aim of the therapy is to improve clinical signs and symptoms, decrease parasitic load and ability to transmit the parasite, and also avoid relapses [21]. Most of drugs are able to reduce parasite load, but none of these eliminate the infection reliably [3,21]. Combination therapy is the best way to avoid parasite resistence, increase treatment efficacy and reduce length of the treatment [11]. In humans is well known developed resistance against amphotericin B, pentavalent antimonials and miltefosine [5].

3.6.1. Cutaneous and mucocutaneous leishmaniasis

There are two choices in the treatment of cutaneous leishmaniasis: systemic and local treatment. In cases of mucocutaneous leishmaniasis, the topical therapy is not indicated [9,12].

Systemic treatment

Pentavalent antimony

The first-line therapy for CL, MCL and also for VL is use of pentavalent antimony (Sb) in form of meglumine antimonate or sodium stibogluconate. The mechanism of action of these compounds is multi-factorial, including effects on molecular processes and energy metabolism – they block the production of adenosine triphosphate (ATP) and guanosine triphosphate (GTP), inhibition of fatty acid oxidation, leishmanial glycolysis and influence on macrophage activity of the parasite [3,12,14]. Antimonials can be administered intravenously, intramuscularly and also intralesionally [12].

These drugs are toxic and they cause common, sometimes life-threatening side effects. The most serious are cardiac arrhythmia, prolongation of the QT interval, acute pancreatitis, elevation of pancreatic and liver enzymes and electrolyte abnormalities. This treatment requires careful observation of patients [11,12]. The main problem of pentavalent antimonials is increasing resistance [9].

Pentamidine

Pentamidine interferes with the biosynthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and phospholipids. Adverse effects include hypoglycemia, leukopenia, anemia, nephrotoxicity and cardiotoxicity such as hypotension, heart failure and arrhythmia [12].

Amphotericin B

This polyene with antifungal activity acts by binding to ergosterole membrane in the parasite's cell. Amphotericin B causes the instability of the membrane and alters its permeability [3,12]. There are two types of amphotericin B. Conventional deoxycholate form is used in some areas in India. The treatment with liposomal amphotericin B is more frequent in Europe and the United States and is highly effective and less toxic [9,11]. Amphotericin B has to be administered intravenously [5]. Common side effects of this drug are fever, rigor, chills, nephrotoxicity and hypokalemia [11,12].

Azoles

Azoles, mainly oral ketoconazole and fluconazole, have *in vitro* activity against *Leishmania* spp. but they are not so effective for clinical usage [12].

Local treatment

Local treatment includes use of paromomycin ointment combined with methylbenzethonium chloride or with urea. The first combination is more effective but there is a higher risk of local inflamatory reactions than in paromomycin-urea treatment [9].

For local treatment a topical imiquimod can be also used. It induces the activation of macrophages due to production of cytokines (IL-2, TNF-α and IFN-γ). The main side effect is irritation after the application. Cryotherapy and heat therapy were also used for the treatment of CL [12].

3.6.2. Visceral leishmaniasis

Treatment of visceral leishmaniasis is based on pentavalent antimonials, amphotericin B, miltefosine, paromomycin and pentamidine which are used in therapy of resistant cases of VL. For the treatment of post-kala azar dermal leishmaniasis are effective the pentavalent antimonials and liposomal amphotericin B [9].

Miltefosine

Miltefosine is the first effective orally taken drug with high cure rates. It is an alkylphospholipid and it was developed as an antineoplastic agent [11,21].

It is able to disturb the cell membrane synthesis and signal pathways which leads to apoptosis. Miltefosine is well-tolerated and side effects are not specific, including nausea and vomiting. This drug is contraindicated in pregnancy because of teratogenetic effect [11,21].

3.6.3. Canine leishmaniasis

The major drugs used for the treatment of CanL are pentavalent antimony, amphoterinic B, miltefosine, aminosidine and marbofloxacine. These leishmanicidal therapy is usually combined with parasitostatic drugs, like allopurinol. Combination of allopurinol and meglumine antimonate has been used in most cases and has high efficacy. Miltefosine-alloprinol combination is also clinicaly effective [5,21].

Allopurinol

Allopurinol is an orally active purine analogue with low toxicity. When this drug is incorporated into the parasite's RNA, it inhibits the protein synthesis. It is not nephrotoxic, so it can prevent or to reduce glomelural damage or proteinuria [21].

3.7. Diagnostic methods

Due to large range of clinical signs and symptoms of leishmaniasis, the diagnosis still represents a challenge [7].

There are various confirmatory tests, which can be used for diagnosis of this disease [8,11]. Diagnostic tests are performed for many reasons, such as confirmation of the disease, observing responses to the treatment and for epidemiological studies and control programs (searching the presence of infection in non-endemic countries and avoiding importation of infection into these areas, including transmission of infection from asymptomatic carriers) [3,22].

Diagnostic tests should have high sensitivity and also high specificity. These tests should recognize difference between asymptomatic infection and acute state, and should be simple and easy to perform in laboratories without expensive equipments. Procedures which lead to obtain samples should be non-invasive [7,11].

3.7.1. Direct methods

3.7.1.1. Microscopic examination

This direct parasitological test still remains the first method of choice for conclusive diagnosis of *Leishmania* infection [23]. *Leishmania* amastigotes are detected in tissue smears from bone marrow, spleen, lymph nodes or skin by microscopy [6]. The samples are stained with Giemsa or Leishman stain. Amastigotes are localized intracellulary in monocytes and macrophages. Their cytoplasm appear pale blue with big red nucleus and deep red or violet kinetoplast [6,8].

This method is highly specific, the sensitivity depends on the tissue which is used and parasitic load [22]. Smears from spleen have sensitivity 93.1-98.7%, bone marrow and lymph nodes aspirates show lower sensitivity (52-85%) [6]. In asymptomatic infection, the sensitivity of this technique can be under 30% [3].

A major disadvantage of this method is that samples (especially splenic aspirates) are obtained painfully and there is a risk of serious and fatal bleeding [6,7].

3.7.1.2. Culture

Promastigote form can be isolated in culture medium from infected tissues such as bone marrow or spleen. The culture media which are used can be monophasic or diphasic. The most used monophasic media are Schneider's insect medium, M199 and Grace's medium which are important in increasing parasite number. The diphasic medium is used in cases where it is necessary to convert amastigotes form to promastigotes. The Novy-McNeal Nicolle medium and Tobies medium are the most known. One or two drops of aspirates are required for inoculation of the media. Ideal temperature for incubation of cultures is between 22 and 28°C. Cultures are evaluated once a week [6-8].

This method is 100% specific and reliable. It is rarely used for diagnosis by now because it is time-consuming, results are delayed and also cultures have higher susceptibility to contamination by bacteria, yeast or other fungi species [7,8].

3.7.2. Indirect methods

3.7.2.1. Molecular diagnosis

Molecular biology techniques such as conventional PCR (polymerase chain reaction) and qRT-PCR (quantitative real-time polymerase chain reaction) are powerful tests which are able to detect DNA or RNA of the parasite and are often used for diagnosis of leishmaniasis. Positive results are obtained in all cases (CL, MCL, VL) and specially these methods are frequently used in the confirmation of the infection in cases where immune response is weak, like HIV-VL coinfection [23].

3.7.2.1.1. Polymerase chain reaction

PCR assays have registered huge success due to their high sensitivity and specificity. Therefore, this method is considered to be an effective tool not only for detection of *Leishmania* DNA but also for monitoring parasitemia after the treatment [7,14]. Polymerase chain reaction should also be used in patients without signs, because PCR shows be useful in confirming asymptomatic infection. Blood or tissues of asymptomatic carriers may contain *Leishmania* DNA and it signalizes that they hide infection and can not develop clinical disease [5].

The principle of PCR is to pair complementary bases of DNA, amplification and detection of specific area in genome with the use of specific primers. For detection of the

product is necessary to perform electrophoresis in agarose or polyacrylamide gels. Ethidium bromide, SYBR Green or silver nitrate are used as a pigments [24]. For detection of *Leishmania* DNA in blood, biological fluids, bone marrow, lymph node, spleen, skin, conjunctiva or buffy coat samples could be used [5].

The PCR is based on diverse target sequences which use ribosomal RNA (rRNA) gene, miniexon genes, nuclear sequences or kinetoplast DNA (kDNA) minicircles or maxicircles [5,6,25,26]. The kinetoplast includes many specific DNA sequences which are in form of maxicircles and minicircles [26]. Kinetoplast DNA is presented in more than ten thousand copies per parasite, in contrast, *Leishmania* parasites contain 40-200 copies of rRNA gene [3]. The amplification of kDNA seems to be the most sensitive and effective target for PCR diagnosis [25]. The biological material, choice of the primers, number of copies of the target method which are used for extraction of DNA and PCR protocol are the main factors which can influence the specificity and sensitivity of the PCR [7,14].

The PCR is more sensitive than microscopic detection of amastigotes in stained samples or parasite cultures [5,16]. However, this diagnostic method requires specific and expensive equipment. These conditions can limit the use of the PCR in developing countries [23].

3.7.2.1.2. Quantitative real-time polymerase chain reaction

Real-time PCR (qRT-PCR) is a progressive molecular technology used for the detection of extremely low loads of parasites, genetic characterization and quantification of parasites [3,25].

Quantitative RT-PCR allows continuous monitoring of parasite levels and detection of products during their amplification. In contrast with conventional PCR, qRT-PCR has improved sensitivity, is less prone to contamination and time of assay is an hour or less [7,27]. During this method, oligonucteotid probes are labelled with fluorophores and the fluorescence emmited allows the detection of *Leishmania* DNA [24].

3.7.2.2. Serological diagnosis

The serological tests are widely and frequentely used for both human and canine *Leishmania* infections [10]. In leishmaniasis, intense humoral immune response and high levels of specific antibodies were observed [14]. Therefore, methods which are provided, are

based on the presence of specific humoral immune response and detection of anti-*Leishmania* antibodies [6,10].

The diagnosis can be carried out using several serological techniques including the direct agglutination test (DAT), fast agglutination screening test (FAST), indirect fluorescent antibody test (IFAT) and ELISA [10].

These methods are valuable, simple, practical, rapid and useful for early diagnosis [10,24]. Due to excessive humoral immune responses, the sensitivity of these methods is very high. The specificity depends on the antigen which is used [8,21].

In HIV patients, the sensitivity of serological tests (mainly IFAT and ELISA) is very low and infection should be confirmed by other methods, like PCR analysis [8].

Serological assays also have several limitations. First, the cross-reactivity with other protozoans may occure and can give false positive results, especially with *Trypanosoma cruzi* in Central, South and North America, *Ehrlichia canis* or with other *Leishmania* species [5,7]. This disadvantage can be limited using recombinat polypeptides with specific epitopes, like rA2 (recombinant A2 antigen), rK9 (recombinant K9 antigen), rK26 (recombinant K26 antigen) and rK29 (recombinant K29 antigen) [3,5]. Second, after succesful cure when antibody levels decrease, some of them can be still detected for many years [11]. For this reason, serology assays are not good methods for diagnosis of relapses or monitoring patients after the treatment [11,24]. Antibody-based test should be used in combination with other tests because they can not recognize differences between acute disease, asymptomatic infection and relapses [15].

3.7.2.2.1. Direct agglutination test

This serological method has a high clinical accuracy, is reliable, non-invasive, simple, and do not require expensive equipment and is widely performed in most endemic areas [6,11,28]. The direct agglutination test uses promastigotes of *L. donovani* in freeze-dried form or in a suspension as an antigen. The DAT can detect antibodies in the plasma or serum of the infected patients [7,28]. Samples are incubated with antigen in microtitre plates. The agglutination is visible after an overnight incubation [6,11].

In negative samples, the DAT antigen form dark blue spot in the botton of the well. Positive results are obtained when antibodies to *L. donovani* are present. The antigen makes blue film over the all wells [28].

The DAT has shown to be from 88% to 93% sensitive, the specificity is between 70% to 100% [24]. The main disadvantage of DAT is long incubation time (18 hours) and multiple diluting of the samples [6]. The modified version of DAT is the fast agglutination screening test (FAST). The FAST is easy to perform, is faster than DAT, uses higher concentration of parasite and requires only single dilution of sera. The results are evaluated after 3 hours [7,11].

3.7.2.2.2. Indirect fluorescent antibody test

Leishmaniasis can be also evaluated by indirect fluorescent antibody test. This method detects anti-*Leishmania* antibodies with high accuracy and is useful in epidemiological studies and treatment monitoring [7,29]. IFAT uses whole parasite body as antigen and antibodies are labeled with flurochromes. Antigen-antibody reaction is detected by fluorescence microscopy. Positive samples show green fluorescence, negatives samples have matt red coloration [7,24].

IFAT is both qualitative and also quantitative method, it means that it allows to evaluate titres of anti-*Leishmania* antibody and is used for mass screening of infected patients [29]. The specificity of IFAT ranges from 60% to 90% and sensitivity from 68% to 100%. However, this technique requires a high level of skills, expensive laboratory facilities and is necessary to make serial dilutions of serum [7,24].

3.7.2.2.3. Enzyme linked immunosorbent assay

ELISA-based techniques have been used as a valuable serodiagnostic tool for many infectious diseases [8]. ELISA is a method based on detection of specific anti-*Leishmania* antibodies and is able to screen large number of samples in a short period of time [10,23]. This assay is highly sensitive but specificity depends on the antigen which is employed. Soluble promastigote extracts and purified or recombinant proteins can be used as antigens. Promastigote stage of different *Leishmania* species provides total soluble crude antigens which are the most used antigens in this assay [5,10].

In the last years, a lot of recombinant proteins have been described and tested for serodiagnosis of *Leishmania* disease in symptomatic and asymptomatic dogs. Many of them, like rK39 (recombinat K39 antigen), rK28 (recombinat K28 antigen) or *Lic*TXNPx (*Leishmania infantum* cytosolic tryparedoxin peroxidase) have been shown as very useful [10,24,30]. ELISA tests based on recombinant proteins have increased sensitivity and specificity and improved accuracy [24]. The recombinant protein rK39 is considered to be a strong and specific diagnostical marker of disease [7]. This antigen is 39-amino acid encoded with 117 base pairs gene which is expressed in kinesin region in the amastigotes of *Leishmania chagasi* [6,8,10,11]. This protein shows sensitivity and specificity of 100% and 96%. The rK39 is more sensitive for diagnosis of symptomatic CanL and human VL than for asymptomatic CanL [10]. The utility of rK39 has been also demonstrated in HIV patients infected with *Leishmania infantum*. Activity of the disease corelates with the antibody titres of this antigen. Recombinant K39 can be used for monitoring of the therapy and it can be also useful for prediction of the relapse [6,8,31].

The rK39-based immunochromatographic test (ICT) has been developed as a promising rapid strip test in recent years. This dipstick test is commercially available and can be used for serodiagnosis of VL and also for CanL [6,32].

The recombinant protein of the parasite is fixed on a small piece of nitrocellulose membrane and unique immunoglobulins which are presented in the serum of infected patients can recognize this antigen. For detection, specific colloidal gold protein A is used and only one drop of blood is necessary for the realization of this assay [6,24,32]. The presence of a red line in the tested area is considered as positive result [22].

ICT is rapid (results can be read visually in 10 minutes), easy to use due to single-test format, it can screen large number of samples in field conditions and does not require expensive equipment. The sensitivity is between 67-100% and specificity range of 97-100% [7,24,32]. The major disadvantage is that ICT can give positive results in a healthy individuals after long period of the cure [6].

3.7.2.2.4. Flow cytometry

Flow cytometry (FC) is a new reliable, promising method and is frequently used for diagnosis of infectious diseases, such as human leishmaniasis and CVL (canine visceral leishmaniasis) [33,34]. This method is able to count, investigate and separate several

thousand microscopic particles which are suspended in a flow of fluid [7]. The serum of infected patients contains specific anti-*Leishmania* antibodies which could be examined by this method [34].

For FC can be used both forms of live parasites, promastigotes and amastigotes. Live amastigotes, the form present in the mammal, have shown to be more useful target in serodiagnosis of symptomatic and asymptomatic dogs than promastigotes [33].

This method can detect anti-fixed *L. chagasi* antibodies of promastigotes - FC-AFPA-IgG (anti-fixed *Leishmania chagasi* promastigotes IgG antibodies detected by flow cytometry), FC-AFPA-IgG1 (anti-fixed *Leishmania chagasi* promastigotes IgG1 antibodies detected by flow cytometry) and FC-AFPA-IgG2 (anti-fixed *Leishmania chagasi* promastigotes IgG2 antibodies detected by flow cytometry) presented in samples of infected dogs by *L. chagasi* and from vaccinated dogs against CanL [7,35]. Flow cytometry can differentiate the serological profile between these two groups. FC-AFPA-IgG method shows to be 95% sensitive and 100% specific [33,35].

The main advantages of this assay are acurate and rapid analysis with reproducible results, high throughput capacity, possibility of analyte quantification and the potencial for multiplexing [7,34].

3.7.2.2.5. Nanodiagnostics

Nanotechnology is relatively recent and rapidly developing method. The range of nanotechnology use is wide. These methods are not applied only for diagnosis but also for drug delivery, cancer management, tissue imaging, for monitoring and detecting molecular and cellular changes which are connected with the state of the disease [36]. These methods offer increased sensitivity, detection can be fast with reduced costs [37]. For increased sensitivity is necessary interaction between molecule which is analyzed, and particles. This enables detection of the analyte. The first step in the most assays including nanoparticles is the binding of a nanoparticle prone or label to the biomolecule target. This process will produce a specific signal which can be measured as a characteristic signal of the biomolecules [37].

Nanodiagnostics has shown growing potential due to the fact that most biological structures have nanometer size. Nanoparticles have certain properties which are dependent on size and should respect optical and magnetical parameters. Many structures have been

designed and can be used as prones, the most powerful are quantum dots (QDs) and nanoshells [37].

Quantum dots are nanocrystals with high photostability, sensitivity, wide excitation spectra and strong light absorbance. They can be also used as fluorescent markes for biomolecules. QDs are mostly used for cancer diagnosis but also can be conjugated to antibodies. The main problem of the QDs is associated with toxicity to humans [37,38].

Nanoshells are presented by gold nanoparticles. They are able to detect and label small pieces of DNA and proteins, including antibodies [37,38].

Nanotechnologies play an important role in the current diagnostics and other areas and also in the future progression of therapeutic and diagnostic methods. Time will show if the nanodiagnostics can replace other actual diagnostic methods because a lot of aspects need to be assessed [37,38].

4. THE AIM OF THE WORK

In the last decades, several techniques have been developed for diagnosis of canine leishmaniasis [5]. Nevertheless, there is still a need for highly sensitive and specific diagnosis, especially asymptomatic cases of CanL. That´s why we decided to explore ELISA based diagnosis tests.

We investigate the potential of recombinant proteins. In my work, I focus on sensitivity and specificity of six antigens - A, B, C, D, E and F for serological diagnosis. The main aim of this study is to find the perfect combination for an early and accurate diagnosis of symptomatic and asymptomatic cases of CanL.

5. PRACTICAL PART

5.1. Materials and Methods

5.1.1. Samples

For this study, there were used 364 serum samples from female and male domestic dogs of different breeds and ages obtained from Portugal (Pt) and Brazil (Br). These sera were divided according to the clinical signs into 3 groups:

Positives: This group of sera was classified as symptomatic based on clinical signs and positive circulation of the parasite (n(Pt)=62; n(Br)=61).

Negatives: These sera were obtained from seronegative dogs that did not present clinical signs, parasite in circulation and all serological tests were negative (n(Pt)=122; n(Br)=74).

Asymptomatic: Sera were recovered from dogs without clinical signs and parasite in circulation but with at least three seropositive tests (n(Pt)=45).

All samples were stored at -20°C until used.

5.1.2. Antigens used for the enzyme-linked immunosorbent assay

Six recombinant proteins (A, B, C, D, E and F) were used in this study. Each letter corresponds to a different recombinant protein or combination with a certain concentration. All antigens were analyzed on 10% polyacrylamide gels and visualized by staining with Coomassie blue. The protein content of each antigen preparation was determined by the Lowry assay.

5.1.3. Indirect enzyme-linked immunosorbent assay

An indirect ELISA method was performed according to the protocol described elsewhere, with small modifications [10]. Ninety-six-well flat-bottomed microtiter plates were coated with 50 µl coating buffer (0.05M carbonate/bicarbonate buffer, pH 9.6). The concentration of each antigen was determined in range from 1 µg/ml to 6 µg/ml. Final concentrations were: 3µg/ml of A, 5 µg/ml of B, C and E, and 6 µg/ml of D and F.

The plates were incubated overnight at 4°C. The content of the plates was discarted and plates were washed with PBS-Tween (PBS-T) 0.05%.

Next, the plates were blocked with 200 µl/well of PBS-milk 3% for one hour at 37°C and washed with PBS-T 0.05% .

Sera were diluted (1:1500 in PBS-T-milk 1%) and added to the plate in triplicate (100µl/well). After incubation - 30 minutes at 37°C - the plates were washed with PBS-T (0.05%).

Anti-dog IgG conjugated to horseradish peroxidase (100 µl/well, diluted 1:5000 with PBS-T-milk 1%) was added and plates were incubated for 30 minutes in dark.

The plates were washed with PBS-T 0.05% and incubated with 0.5 mg/ml of o-phenylenediamine dihydrochloride (OPD, Sigma), 100 µl/well, for 10 minutes in the dark.

The reaction was stopped with 50 µl/well of 3M HCl and absorbance values were read at 492 nm in an automatic ELISA reader (Bio Tek Instruments, Inc.).

In all experiments, the blank, positive and negative controls were included in each plate as an internal control.

5.1.4. Statistical analysis

Statistical analyses were carried out by using GraphPad Prism 5 (GraphPad Software Inc.). Receiver operating characteristic (ROC) curves were generated for each tested antigen. The sensitivity values are located in the ordinate and complement of specificity in the abscissa. The ELISA cut-off values were defined based on ROC curves, and were used to distinguish positive from negative samples.

6. RESULTS AND DISCUSSION

Based on ROC curves, we determined the diagnostic potencial of six recombinant antigens - A, B, C, D, E and F. We evaluated the following parameters: area under the curve (AUC), cut-off point, sensitivity (Se), specificity (Sp), false negatives (FN) and false positives (FP).

6.1. Determination of cut-off point and AUC

The ELISA cut-off values for all antigens were determinated on the basis of ROC curves. A ROC curve was constructed for each antigen [10], separately, for samples from Portugal and from Brazil. For both regions, groups of positive and negative samples were used in this determination, and moreover, for portuguese samples, a second ROC curve with asymptomatic and negatives sample was determined.

AUC is an indicator of how useful the test is and it is also used to compare accuracies of different diagnostic tests or antigens. On the one hand, when AUC is equal to 1, the test is considered perfect, and on the other hand, if AUC is 0.5 or less, the test is considered to be useless. To demonstrate excellent accuracy, the AUC should be greater than 0.97 [10,39,40].

6.1.1. Determination of AUC and cut-off point for positive and negative samples

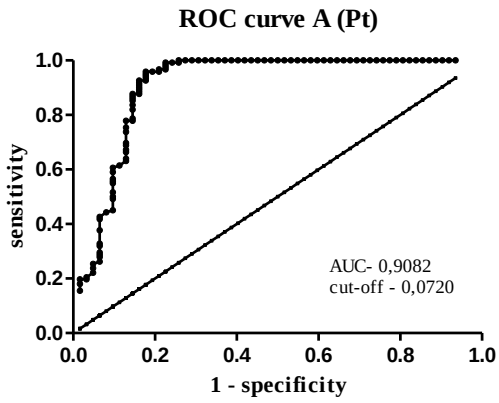
For Portugal samples, the AUC value obtained for antigen A was 0.9082 with a confidence interval (CI) (95%) between 0.8512 to 0.9653. These results show reduced accuracy, below 0.97 in comparison with Santarem et al. [10]. The rest of antigens showed AUC higher than 0.97 as is figured in the table 1. Primising shows to be antigen C with AUC 0.9966 and CI (95%) between 0.9925 to 1.001 (Figs.6-8)

For samples from Brazil, the lowest AUC values were noticed for antigen A and B, below 0.97. Area under the curve for antigens B, C and F have the same value (0.9911) but different confidence interval. Based on these results, antigen C has showed excellent results with very high AUC values for both, Portugal and Brazil samples (Table 1.).

Table 1. Area under the curve (AUC) and confidence interval (CI) for positive and negative samples.

Portugal						
antigen	A	B	C	D	E	F
AUC	0.9082	0.9718	0.9966	0.9833	0.9783	0.9948
CI 95%	[0.8512; 0.9653]	[0.9522; 0.9914]	[0.9925; 1.001]	[0.9662; 1.000]	[0.9594; 0.9972]	[0.9890; 1.001]
Brazil						
antigen	A	B	C	D	E	F
AUC	0.9348	0.9911	0.9911	0.9438	0.9760	0.9911
CI 95%	[0.8846; 0.9849]	[0.9811; 1.001]	[0.9759; 1.006]	[0.8951; 0.9926]	[0.9556; 0.9963]	[0.9801; 1.002]

I.



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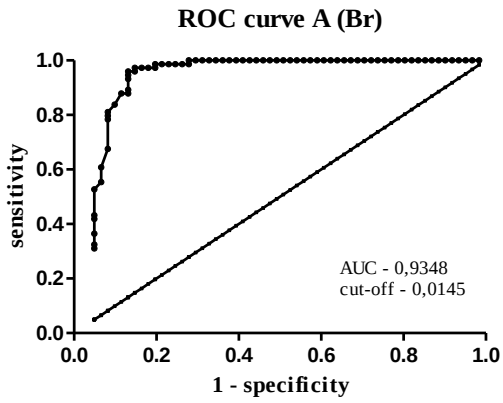

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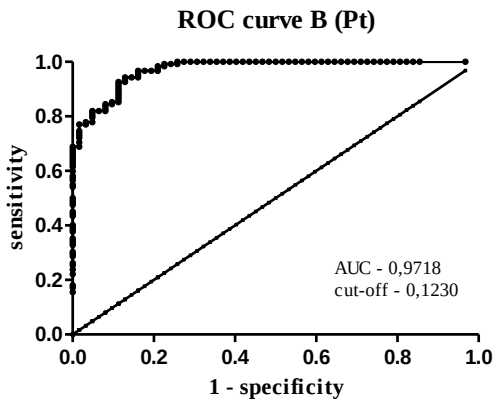

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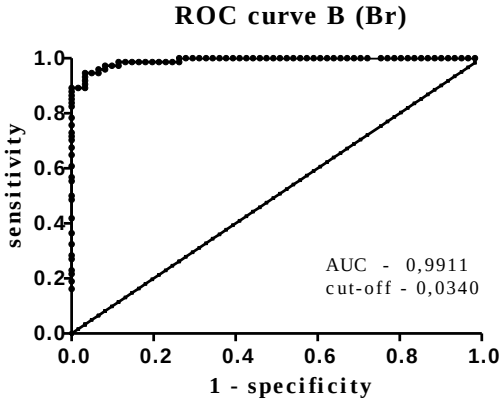
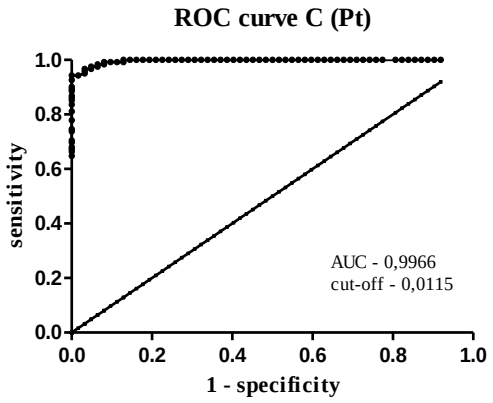


Fig. 6 ROC curves for antigen A and B constructed with positive and negative samples: (I.) antigen A – Pt samples; (II.) antigen A – Br samples; (III.) antigen B - Pt samples; (IV.) antigen B - Br samples

I.



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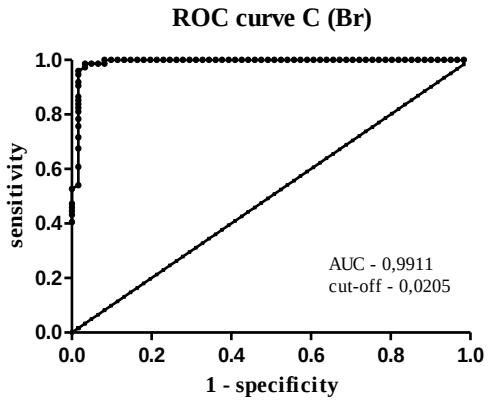

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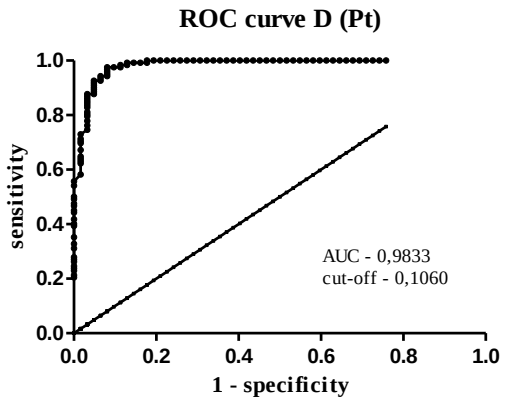

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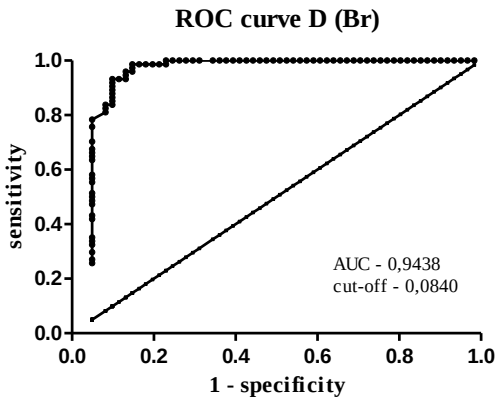
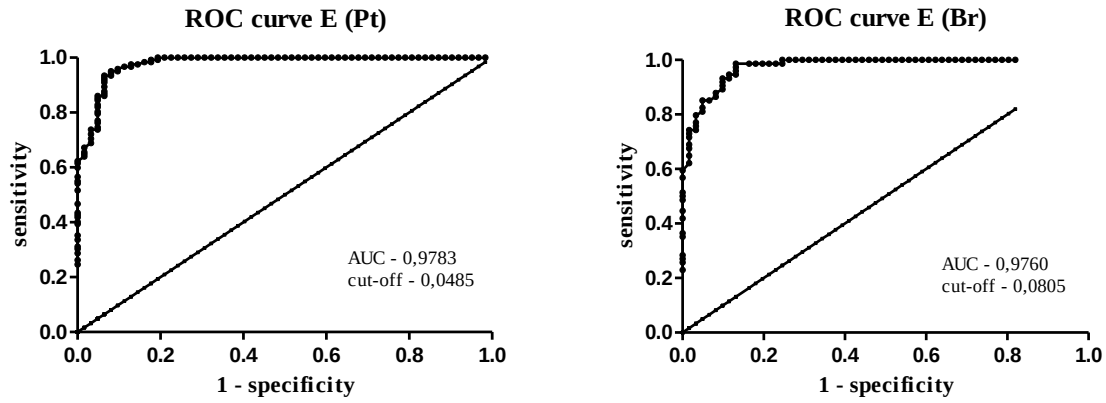


Fig. 7 ROC curves for antigen C and D constructed with positive and negative samples: (I.) antigen C – Pt samples; (II.) antigen C – Br samples; (III.) antigen D – Pt samples; (IV.) antigen D – Br samples.

I. **II.**



III. **IV.**

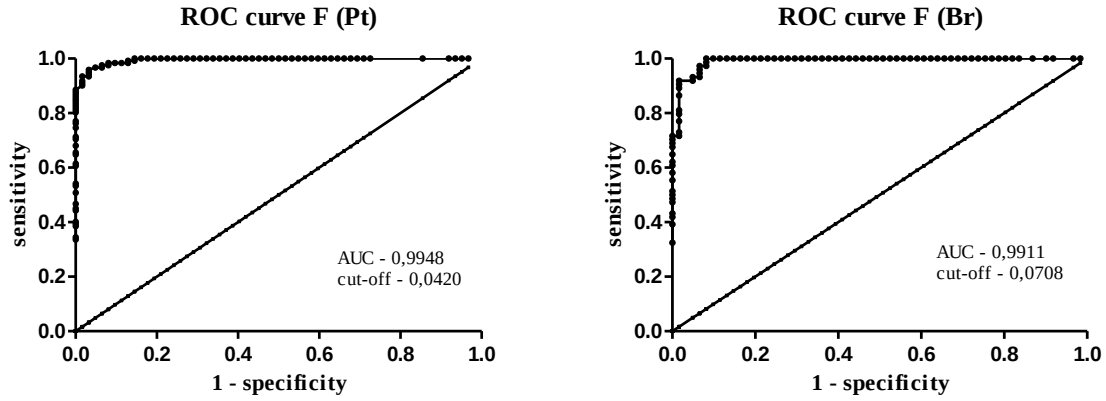


Fig. 8 ROC curves for antigen E and F constructed with positive and negative samples: (I.) antigen E – Pt samples; (II.) antigen E - Br samples; (III.) antigen F - Pt samples; (IV.) antigen F - Br samples.

Based on ROC curves, we also determined the ELISA cut-off values (optical density at 492nm) for all formulations. The lower cut-off values were found for antigen C in both studied countries. However, the antigen D has the highest cut-off namely for portuguese samples. The values are described in the next section in the table 3.

6.1.2. Determination of AUC and cut-off point for asymptomatic and negative samples

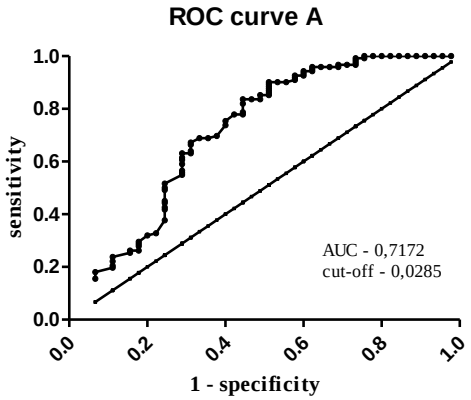
Area under the curve was determined for asymptomatic and negative samples by the same way as for positive and negative samples. Obtained AUC were classified as excellent only for antigen C with AUC 0.9728 and CI (95%) between 0.9388 to 1.007. In the table 2. are noticed results for other antigens but they reached lower values . By comparing the AUC for symptomatic and asymptomatic samples, there were found lower values for the last, as we expected.

Table 2. Area under the curve (AUC) and confidence interval (CI) for asymptomatic and negative samples.

Portugal						
antigen	A	B	C	D	E	F
AUC	0.7172	0.8742	0.9728	0.9543	0.8878	0.9686
CI 95%	[0.6191; 0.8153]	[0.8125; 0.9360]	[0.9388; 1.007]	[0.9179; 0.9907]	[0.8230; 0.9526]	[0.9395; 0.9977]

The lowest cut-off value was obtained for antigen C and highest for antigen D. ELISA cut-off points (optical density at 492nm) for asymptomatic samples are listed in the table 4. In general, the cut-offs of the group of asymptomatic and negative samples were lower then the symptomatic, as was expected due to the lower reactivity of that group (Fig.9)

I.



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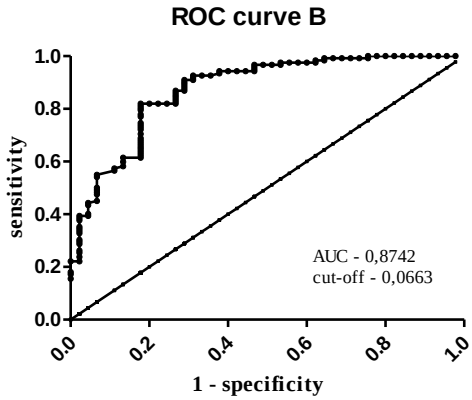

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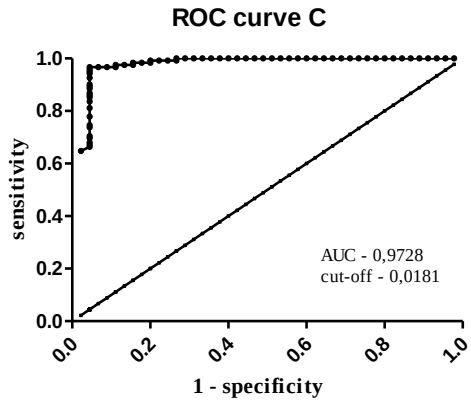

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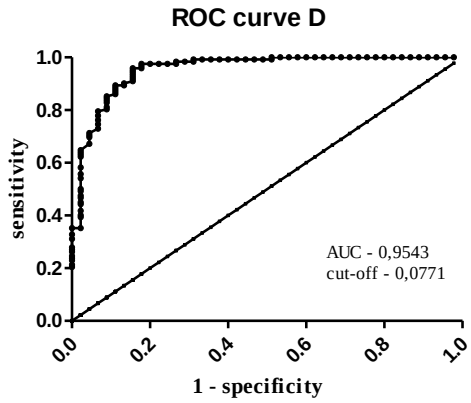

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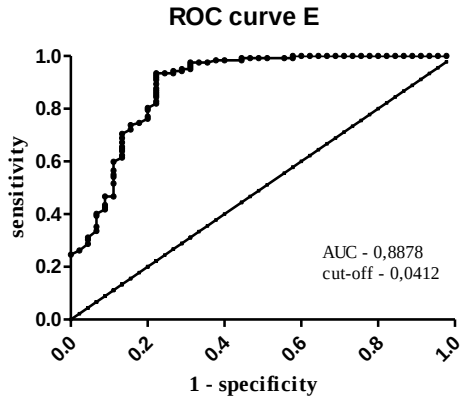

VI.

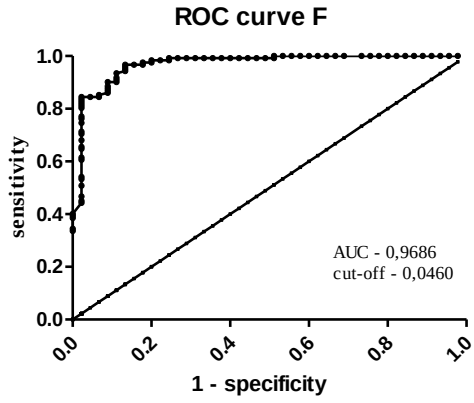

Fig. 9: ROC curves for antigen A, B, C, D, E and F constructed with Pt asymptomatic and negative samples: (I.) antigen A ; (II.) antigen B; (III.) antigen C; (IV.) antigen D; (V.) antigen E; (VI.) antigen F.

6.2. Determination of ELISA sensitivity and specificity

In this study, we also tested sensitivity and specificity of each antigen using positive and negative dog serum samples, and asymptomatic and negative samples. The specificity was defined by evaluating the number of false positives obtained against sera from *Leishmania*-negative dogs.

6.2.1. Determination of ELISA sensitivity and specificity for positive and negative samples

Antigen A did not show high potencial in the diagnosis of CanL because it was not able to recognize 11 Pt and 8 Br positive samples. Sensitivity values are low for both, Pt and Br dog samples, and specificity values are identic (table 3). Higher difference was registered in the sensitivity of antigen B, for Pt samples (54 out of 62) and Br samples (59 out of 61), the specificity values were almost the same.

Antigen C presented the best sensitivity in the diagnosis of CanL for Pt samples. The specificity values were different for Pt (115 out of 122) and Br (73 out of 74) samples compared with antigen A and B, where values were very similar for both regions. Small differences in sensitivity were registered for antigen D and E in Br samples. In both cases, sensitivity had the same value for Pt samples (57 out of 62). Antigen D and E successfully detected negative Pt samples (D - 119 out of 122 and E - 116 out of 122). Specificity for Br samples was also the same for both antigens (98.64%).

Table 3. Characterization of the formulations using positive sera

	A		B		C		D		E		F	
	Pt	Br	Pt	Br	Pt	Br	Pt	Br	Pt	Br	Pt	Br
AUC	0.9082	0.9348	0.9718	0.9911	0.9966	0.9911	0.9833	0.9438	0.9783	0.976	0.9948	0.9911
cut-off	0.072	0.0145	0.123	0.034	0.0115	0.0205	0.1060	0.0840	0.0485	0.0805	0.0420	0.0798
Se	51/62 (82.3%)	53/61 (86.8%)	54/62 (87.1%)	59/61 (96.7%)	62/62 100%	59/61 (96.7%)	57/62 (91.9%)	52/61 (84.2%)	57/62 (91.9%)	53/61 (86.9%)	60/62 (96.8%)	56/61 (91.8%)
Sp	117/122 (95.9%)	71/74 (95.9%)	115/122 (94.3%)	70/74 (94.6%)	115/122 (84.3%)	73/74 (98.6%)	119/122 (97.5%)	73/74 (98.6%)	116/122 (95.1%)	73/74 (98.6%)	117/122 (95.9%)	74/74 (100%)
FP	4.1%	4.1%	5.7%	5.4%	5.5%	1.4%	2.5%	1.4%	4.9%	1.4%	4.1%	0%
FN	17.7%	13.1%	12.9%	3.3%	0%	3.2%	8.1%	14.8%	8.1%	13.1%	3.2%	8.2%

Area under the curve (AUC), cut-off point, sensitivity (Se) and specificity (Sp) of A, B, C, D, E and F antigens based on ROC curve of positive and negative samples.

Finally, antigen F was 100% specific for Br samples, and only 5 Pt samples were not recognized. Antigen F displayed response higher than 90% in the recognition of positives Pt and Br samples: 96.77% and 91.80%, respectivelly.

Some different recombinant antigens like LAM (*Leishmania* antigen mixture), rK39, *Lic*TXNPx (*Leishmania infantum* cytosolic tryparedoxin peroxidase), *Lim*TXNPx (*Leishmania infantum* mitochondrial tryparedoxin peroxidase) and total parasite extracts have been tested earlier. The LAM (combination of *Lic*TXNPx 1 µg/ml and rK39 4 µg/ml) demonstrated higher sensitivity values for positive sera (96.4%) than *Lic*TXNPx and rK39 alone with specificity of 96.3%. According to this study, LAM was considered an improved diagnostic marker which is able to detect clinical and subclinical form of CanL with very high levels of sensitivity and specificity [10]. In comparison with LAM, sensitivity levels for Pt positives samples for antigen C and F were higher. Lower values were detected for the rest of antigens: A, B, D and E.

6.2.2. Determination of ELISA sensitivity and specificity for asymptomatic and negative samples

Considering the results previously described for the group of symptomatics and negatives, once again antigen C and F present the best results for the asymptomatic and negative group, even this comparison is only observed in Portugal samples (Table 4).

Table 4. Characterization of the formulations using assymptomatic cases

	A	B	C	D	E	F
	Pt	Pt	Pt	Pt	Pt	Pt
AUC	0.7172	0.8742	0.9728	0.9543	0.8878	0.9686
cut-off	0.0285	0.0663	0.0181	0.0771	0.0412	0.0460
Se	25/45 (55.6%)	37/45 (82.2%)	43/45 (95.6%)	38/45 (84.4%)	35/45 (77.8%)	39/45 (86.7%)
Sp	102/122 (83.6%)	103/122 (84.4%)	118/122 (96.7%)	117/122 (95.9%)	115/122 (94.3%)	118/122 (96.7%)
FP	16.4%	15.6%	3.3%	4.1%	5.7%	3.3%
FN	44.5%	17.8%	4.5%	15.7%	22.2%	12.3%

Area under the curve (AUC), cut-off point, sensitivity (Se) and specificity (Sp) of A, B, C, D, E and F antigens based on ROC curve of asymptomatic and negative samples.

Antigen A failed not only in detection of positives samples but also in failure to recognize asymptomatic samples. Obtained sensitivity and specificity values are lower (Se = 55.6%, Sp = 83.6%). Therefore, antigen A did not proved to be a good marker for detection

of asymptomatic cases. Similar values in sensitivity (82.2%) and specificity (84.4%) were obtained for antigen B. Antigen C was evaluated as the best for detection of asymptomatic samples. It showed the highest sensitivity and specificity. It was able to recognize 43 from 45 asymptomatic samples and 118 from 122 negatives samples. The same specificity was registered for antigen F (96.7%) but sensitivity is lower (86.7%). Depending on these results, antigen F was the second best. Specificity values for antigen D and E were above 90% but antigen E showed lower sensitivity than antigen D (35 out of 45 and 38 out of 45).

In recent study from Santarem et al. [10], sensitivity and specificity of ELISA method were tested also for asymtomatic samples with using different recombinant antigens. Antigen LAM displayed the best level of sensitivity (82.4%) and specificity (96.3%) in detecting asymptomatic samples [10]. Our work shows lower sensitivity for antigen A, B and E than LAM. Nevertheless, the most useful antigen for diagnosis of subclinical form could be protein C that showed improved sensitivity of almost 13% (95.5%). Antigen D and F did not show bigger differences in sensitivity compared with LAM.

6.3. Evaluation of the reactivity of antigen A, B, C, D, E and F in the Pt and Br CanL population

The results were expressed at the optical density at 492 nm. The reactivity was measured for sera of positive and negative dogs from Portugal and Brazil (Figs.10,11), and for asymptomatic and negative dogs from Portugal (Fig.12). The sera of Pt symptomatic dogs were found to be more reactive against protein F and D than B, C and E. Also asymptomatic samples were the most reactive against antigen F. The lowest reactivity was showed by protein A in both symptomatic and asymptomatic Pt and Br sample groups. The sera of Br symptomatic dogs were more reactive agains antigen F. Antigen E and D showed similar reactivity.

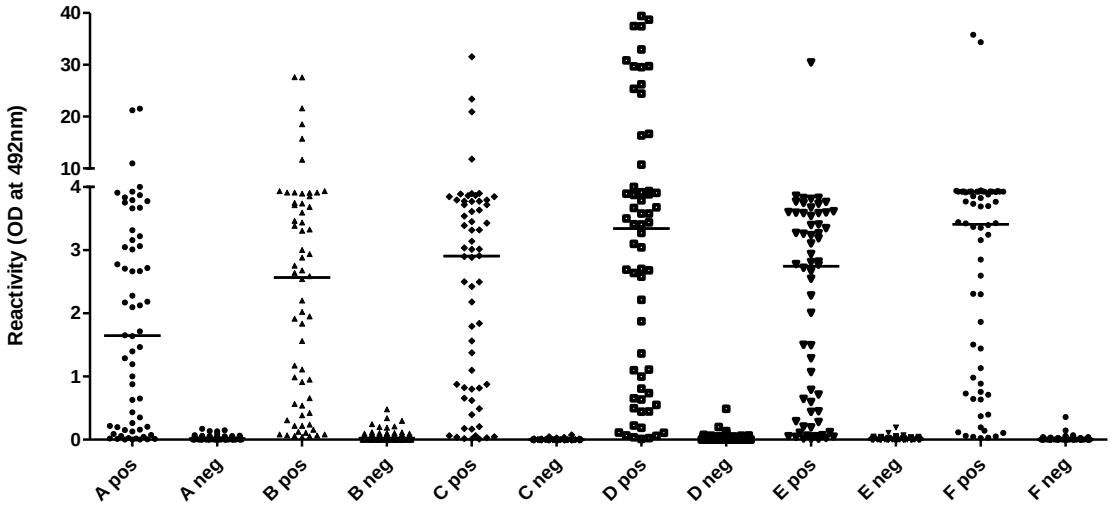

Fig. 10 The reactivity of Pt positive and negative sera against protein A, B, C, D, E and F.

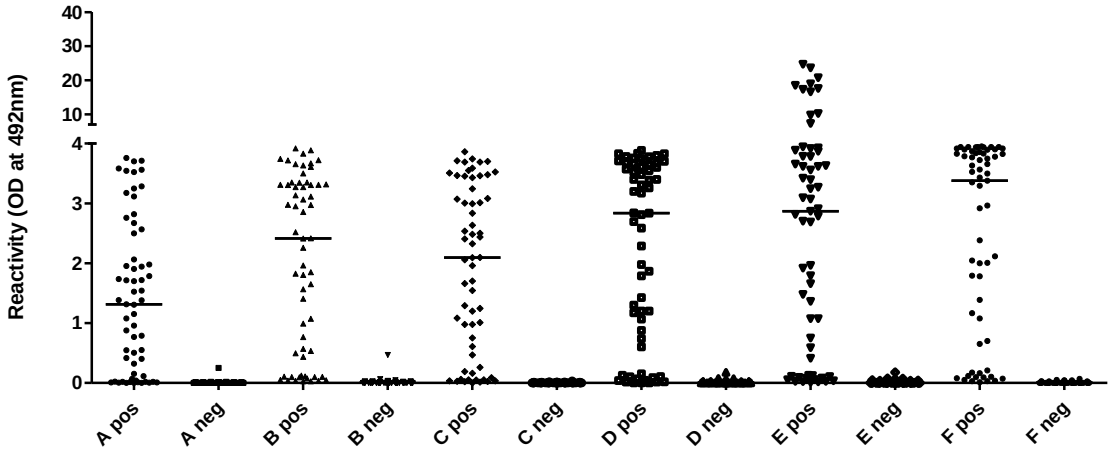

Fig. 11 The reactivity of Br positive and negative sera against protein A, B, C, D, E and F.

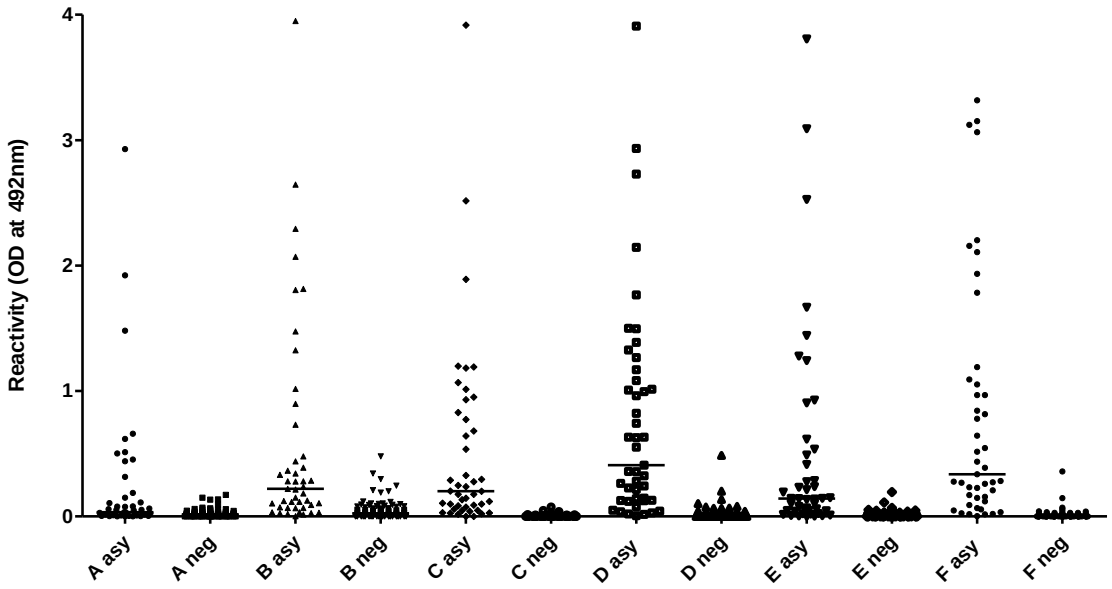

Fig. 12 The reactivity of Pt asymptomatic and negative sera against protein A, B, C, D, E and F.

The reactivities of symptomatic and asymptomatic Pt sera were tested for SPLA (soluble promastigote *Leishmania* antigens), SALA (soluble amastigote *Leishmania* antigens), LicTXNPx, LimTXNPx, rK39 and LAM in some recent studies [10]. In our work, in positives samples all recombinant proteins except antigen A showed higher reactivity than the LAM described by Santarem et al. [10]. Nevertheless, the reactivity of asymptomatic sera against LAM was higher than antigen A, B, C, D, E and F.

7. CONCLUSION

The development of diagnostic methods for detection of leishmaniasis is a relevant step to control this zoonosis. Special attention should be given to asymptomatic carriers which are frequently complicated to detect.

This study describes evaluation of six formulations. It should help to find the best antigen for the accurate diagnosis of positive and mainly asymptomatic cases of CanL. After comparison of all results obtained from ELISA assays, we selected the best recombinant protein: antigen C.

This study displays that antigen C demonstrate high sensitivity and specificity for diagnosis of dogs infected with CanL. Only antigen C was capable to recognize asymptomatic samples with very high sensitivity (95.6%) and specificity (96.7%). Nevertheless, the study noticed good performance of antigen F and D in detecting of symptomatic and asymptomatic *Leishmania* infection in Portugal and Brazil.

For more accurate specification is necessary to confirm these results with larger scale of the dog samples by ELISA and as well with other method with higher sensitivity and specificity, such as flow cytometry.

The role of the recombinant proteins in ELISA method for the serological diagnosis is very important and interesting to explore. These antigens could help in testing asymptomatic samples and they can also reduce the cross-reactivity and provide new diagnostic approach.

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10. REFERENCES

1. Moore EM, Lockwood DN (2011) Leishmaniasis. Clin Med 11: 492-497.

2. Roberts MT (2005) Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. Br Med Bull 75-76: 115-130.

3. Miro G, Cardoso L, Pennisi MG, Oliva G, Baneth G (2008) Canine leishmaniosis--new concepts and insights on an expanding zoonosis: part two. Trends Parasitol 24: 371-377.

4. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L (2008) Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol 24: 324-330.

5. Solano-Gallego L, Koutinas A, Miro G, Cardoso L, Pennisi MG, et al. (2009) Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet Parasitol 165: 1-18.

6. Srivastava P, Dayama A, Mehrotra S, Sundar S (2011) Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg 105: 1-6.

7. Maia C, Campino L (2008) Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet Parasitol 158: 274-287.

8. Sundar S, Rai M (2002) Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol 9: 951-958.

9. Piscopo TV, Mallia AC (2006) Leishmaniasis. Postgrad Med J 82: 649-657.

10. Santarem N, Silvestre R, Cardoso L, Schallig H, Reed SG, et al. (2010) Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. J Clin Microbiol 48: 1866-1874.

11. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, et al. (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 5: 873-882.

12. McGwire BS, Satoskar AR (2014) Leishmaniasis: clinical syndromes and treatment. QJM 107: 7-14.

13. Esch KJ, Petersen CA (2013) Transmission and epidemiology of zoonotic protozoal diseases of companion animals. Clin Microbiol Rev 26: 58-85.

14. Alvar J, Canavate C, Molina R, Moreno J, Nieto J (2004) Canine leishmaniasis. Adv Parasitol 57: 1-88.

15. Ready PD (2010) Leishmaniasis emergence in Europe. Euro Surveill 15: 19505.

16. Ready PD (2014) Epidemiology of visceral leishmaniasis. Clin Epidemiol 6: 147-154.

17. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, et al. (2009) Systemic and compartmentalized immune response in canine visceral leishmaniasis. Vet Immunol Immunopathol 128: 87-95.

18. Cardoso L, Schallig HD, Cordeiro-da-Silva A, Cabral M, Alunda JM, et al. (2007) Anti-Leishmania humoral and cellular immune responses in naturally infected symptomatic and asymptomatic dogs. Vet Immunol Immunopathol 117: 35-41.

19. Coelho EA, Ramirez L, Costa MA, Coelho VT, Martins VT, et al. (2009) Specific serodiagnosis of canine visceral leishmaniasis using Leishmania species ribosomal protein extracts. Clin Vaccine Immunol 16: 1774-1780.

20. Otranto D, Dantas-Torres F (2013) The prevention of canine leishmaniasis and its impact on public health. Trends Parasitol 29: 339-345.

21. Noli C, Saridomichelakis MN (2014) An update on the diagnosis and treatment of canine leishmaniosis caused by Leishmania infantum (syn. L. chagasi). Vet J 202: 425-435.

22. Silva DT, Starke-Buzetti WA, Alves-Martin MF, Paixao Mdos S, Tenorio Mda S, et al. (2014) Comparative evaluation of several methods for Canine Visceral Leishmaniasis diagnosis. Rev Bras Parasitol Vet 23: 179-186.

23. Silvestre R, Santarem N, Teixeira L, Cunha J, Schallig H, et al. (2009) Evaluation of Leishmania species reactivity in human serologic diagnosis of leishmaniasis. Am J Trop Med Hyg 81: 202-208.

24. Veras PST, Fraga DBM, Solcà MdS, Guedes CES (2014) New Advances in the Diagnosis of Canine Visceral Leishmaniasis - Trends in Epidemiology, Diagnosis and Treatment, Dr. David Claborn (Ed.), ISBN: 978-953-51-1232-7

25. Antinori S, Calattini S, Piolini R, Longhi E, Bestetti G, et al. (2009) Is real-time polymerase chain reaction (PCR) more useful than a conventional PCR for the clinical management of leishmaniasis? Am J Trop Med Hyg 81: 46-51.

26. Weirather JL, Jeronimo SM, Gautam S, Sundar S, Kang M, et al. (2011) Serial quantitative PCR assay for detection, species discrimination, and quantification of Leishmania spp. in human samples. J Clin Microbiol 49: 3892-3904.

27. Reithinger R, Dujardin JC (2007) Molecular diagnosis of leishmaniasis: current status and future applications. J Clin Microbiol 45: 21-25.

28. Adams ER, Jacquet D, Schoone G, Gidwani K, Boelaert M, et al. (2012) Leishmaniasis direct agglutination test: using pictorials as training materials to reduce inter-reader variability and improve accuracy. PLoS Negl Trop Dis 6: e1946.

29. Otranto D, Paradies P, Sasanelli M, Spinelli R, Brandonisio O (2004) Rapid immunochromatographic test for serodiagnosis of canine leishmaniasis. J Clin Microbiol 42: 2769-2770.

30. Souza AP, Soto M, Costa JM, Boaventura VS, de Oliveira CI, et al. (2013) Towards a more precise serological diagnosis of human tegumentary leishmaniasis using Leishmania recombinant proteins. PLoS One 8: e66110.

31. Houghton RL, Petrescu M, Benson DR, Skeiky YA, Scalone A, et al. (1998) A cloned antigen (recombinant K39) of Leishmania chagasi diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. J Infect Dis 177: 1339-1344.

32. Otranto D, Paradies P, Sasanelli M, Leone N, de Caprariis D, et al. (2005) Recombinant K39 dipstick immunochromatographic test: a new tool for the serodiagnosis of canine leishmaniasis. J Vet Diagn Invest 17: 32-37.

33. Silvestre R, Santarem N, Cunha J, Cardoso L, Nieto J, et al. (2008) Serological evaluation of experimentally infected dogs by LicTXNPx-ELISA and amastigote-flow cytometry. Vet Parasitol 158: 23-30.

34. Sousa S, Cardoso L, Reed SG, Reis AB, Martins-Filho OA, et al. (2013) Development of a fluorescent based immunosensor for the serodiagnosis of canine leishmaniasis combining immunomagnetic separation and flow cytometry. PLoS Negl Trop Dis 7: e2371.

35. Andrade RA, Silva Araujo MS, Reis AB, Gontijo CM, Vianna LR, et al. (2009) Advances in flow cytometric serology for canine visceral leishmaniasis: diagnostic applications when distinct clinical forms, vaccination and other canine pathogens become a challenge. Vet Immunol Immunopathol 128: 79-86.

36. McNeil SE (2005) Nanotechnology for the biologist. J Leukoc Biol 78: 585-594.

37. Azzazy HM, Mansour MM, Kazmierczak SC (2006) Nanodiagnostics: a new frontier for clinical laboratory medicine. Clin Chem 52: 1238-1246.

38. Jain KK (2007) Applications of nanobiotechnology in clinical diagnostics. Clin Chem 53: 2002-2009.

39. Jones CM, Athanasiou T (2005) Summary receiver operating characteristic curve analysis techniques in the evaluation of diagnostic tests. Ann Thorac Surg 79: 16-20.

40. Akobeng AK (2007) Understanding diagnostic tests 3: Receiver operating characteristic curves. Acta Paediatr 96: 644-647.