Experimental infection of canine from Khartoum State with
*Leishmania donovani*

By

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Dedication

To my parents. My brothers. My sisters. All my friends and colleagues.
ACKNOWLEDGMENTS

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المستخلص

هدفت هذه الدراسة لمعرفة أما إذا كانت الليشمانيا الحشوية يمكن أن تصيب الكلاب وتظهر عليها أعراض مرض ليشمانيا الكلاب الحشوية (Canine Visceral Leishmaniasis)؛ وكذلك هدفت التجربة لدراسة دور الكلاب المحلي (Canis familiaris) في انتشار مرض اللمانية الحشوية.


تم عدد عدد 17 كلاً عن طريق الوريد بالطور المسطح لمرض اللمانيا الحشوية بجرعة تركزها (9 × 4). حفظ عدد 7 كلاً دون عدد كعيبة مرجعية.

استمرت معينة الكلاب شهراً للكشف عن ظهور أعراض مرض ليشمانيا الكلاب الحشوية. أخذت عينات دم من الوريد وسحب سائل الغدد الليمفاوية، بغض النظر عن ظهور أعراض مرضية باستخدام تقنية الكشف المجهرية والمقتنيات الطيفية. استخدم جهاز Polymeerase Chain Reaction (PCR) لتحليل الدم، أظهرت الكلاب التي تم حقنها بالطيف أعراض مرضية مطابقة للأعراض الإكلينيكية لمرض ليشمانيا الكلاب الحشوية، مثل: تضخم العقد الليمفاوية في 13 كلاً (76.5%) وتضخم المغزلي في 7 كلاً (41.2%) والفيل في المخالب في 10 كلاً (58.8%). وقد ظهرت هذه الأعراض بعد مضي شهرين من العدوى بالطور المسطح.

كما أوضح الكشف عن الطفيل نتائج إيجابية بشرائح مسحات السائل الليمفاوي (Promastigotes) في ثلاثة من الكلاب المصابة (17%) وذلك بعد مضي ثلاثة أشهر من العدوى.

أوضح نتائج مسحات الدم ظهور الأنيميا من النوع (Normocytic - Hypochromic) في 12 من الكلاب المصابة (70.5%) بعد ثلاثة أشهر من العدوى.

استخدمت في التجربة عدد 4 بانوات (Primer pairs) بغرض إيجاد الطريقة المثلى للكشف عن المرض. اثنان من البانوات استهدفت الجينوم (Pia1-Pia2 and Pia3-Pia4) والأخرى المادة (RV1-RV2 and AJS3-DB8) في الميتوكندرية (Kinetoplast) وخصوصية كل من الطرقتين وقد تبين أن كل منهما له الفرق على الكشف عن وجود الطبيل في كل العينات المأخوذة من الحيوانات المصابة. إلا أن هناك تباين في حساسية البانات، حيث أن الباندة (RV1-RV2) أعطت نتائج إيجابية بنسبة 100% في الحيوانات المصابة بحزمة حجمها 145 قاعدة نيتروجينية بعد مضي 16 شهراً من العدوى مما يؤكدا وجود الطبيل.

توضح نتائج هذه التجربة أن الكلاب التي تم جمعها من منطقة خالية من مرض اللمانيا الحشوية يمكن أن يصاب بالمرض خلال فترة حضانة تتراوح ما بين 14 – 16 شهراً بعد العدوى.
ABSTRACT

The objectives of this study were directed to find out if *Leishmania donovani* can infect and cause disease in dogs (Canine Visceral Leishmaniasis) and to investigate the role of dogs (*Canis familiaris*, Linnaeus, 1758) as reservoir and/or host of Visceral Leishmaniasis.

The study attempted an experimental infection of some indigenous dogs with *Leishmania donovani* to know the likelihood of infection and sequentially the onset of the canine visceral disease (CVL). The study was executed in a non common area of VL (Khartoum State) from Dec 2006 to Dec 2008. All the 24 puppies (randomly collected) were immunized and vaccinated against the familiar endemic infections (e.g. Rabies, Canine contagious hepatitis, Canine parvovirus disease and Respiratory contagion). For the experimental trail 17 dogs were inoculated intravenously with a dose of $4 \times 10^8$ promastigotes of *Leishmania donovani* while the remaining 7 dogs were incorporated as the control group. Dogs were inspected for the development of indications and signs of CVL on monthly basis. Venous blood and lymph node aspirate samples were collected for the detection of infection using the PCR, microscopy and culture. The Sysmex machine (model CTH- 21) was applied for recording the haematological parameters. The typical clinical signs of CVL started to come to pass within two months post infection. Lymphadenomegaly was observed in 13 dogs (76.5%), Onychogryposis in 10 dogs (58.8%) and Splenomegaly in 7 dogs (41.2%). Parasitological examination showed the presence of the distinctive *Leishmania* amasigotes in the lymph node of three dogs (17%) within 3 months post infection. The haematological findings showed that 12 out of 17
dogs (70.5%) had anaemia. The blood parameters (Blood cells count, Haemoglobin estimation, and Packed Cells Volume value) showed significant differences when compared to the samples obtained from the control group (p<0.05). *Leishmania donovani* DNA was traced using the primer pair (RV1-RV2) in all the experimentally infected dogs16 months post infection. The outcome of this study showed the liability of dogs to be infected with *Leishmania donovani*, and thus can be considered as a potential reservoir of leishmaniasis.
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CHAPTER ONE

INTRODUCTION

According to the World Health Organization (WHO, 1990), leishmaniasis is considered as one of the most important parasitic diseases. It is endemic in 88 countries in five continents with 12 million people afflicted worldwide from it and 350 million living at risk of infection. About 1-1.5 million new cases of cutaneous leishmaniasis (CL) and 500,000 of visceral leishmaniasis (VL) occur each year (Desjeux, 1996). The disease is attributed to Leishmania, haemoflagellate protozoan parasites of the Trypanosomatidae family. In many localities more than one Leishmania species co-exist with overlapping animal hosts and vectors. It is a zoonotic disease but it is still not known to what extent that affects the epidemiology, transmission and planning of control measures. During the recent years parallel to the development of techniques based on the genomic information of organisms, there is an increased effort to apply this knowledge to disease diagnostics. In the course of recent years, some methods were developed for the diagnosis of leishmaniasis based on the genomic information of the parasite. Some of them could detect the parasite very successfully, some not and some are still standing under question.

Kala-azar is one of the most important parasitic tropical diseases in Sudan (WHO, 1990) and the country is considered to be one of the most important foci in the world (Osman et al. 2000). Visceral Leishmaniasis has been described in Sudan since the beginning of the 20th century (Neave, 1904),
with the first important epidemics reported by Stephenson (1940) in the Upper Nile Province and Fung area in 1936–38.

Zoonotic transmission of the parasites is well established in the case of *Leishmania infantum* in Mediterranean countries. In East Africa, the transmission of the dominant parasite species causing VL, *Leishmania donovani* is thought to be anthroponotic (Desjeux, 2001).

Canine Visceral Leishmaniasis (CVL) is a common infectious disease in the countries of the Mediterranean basin. The disease has variable clinical pictures, where almost every system is involved. This may include progressive loss of body weight, cutaneous ulcers, peripheral lymphadenomegaly, and epistaxis. Studies in European foci have shown that the prevalence of CVL varies from 1 to 37% (Alvar *et al*., 2004).

However, the finding of infected *Phlebotomus orientalis* (3.5 - 7.1% of flies infected) in the uninhabited Dinder Natural Park strongly suggest the presence of a reservoir other than man. This is supported by recent findings of possible *L. donovani* infections in dogs and some other animals (Mukhtar *et al* 2000; Dereure *et al*., 2000; El-Naiem *et al*., 2001).

Because of lack of knowledge on the reservoir host of *L. donovani*, few attempts were made to describe the transmission cycles of Visceral Leishmaniasis. The findings by the NAMRU-3 team, Hoogstraal & Heynemann (1969) presented a general model for possible transmission cycles of VL in the area. In this model the principal zoonotic cycle occurs in the Acacia-Blalanites forests.
Kirk (1939) examined many animals but a part from some casual infections in a monkey, a horse and a fox he found no evidence for incrimination of a reservoir host. Similarly, the NAMRU-3 team in the Upper Nile Province found few infected animals, but no conclusions were drawn on reservoir hosts (Hoogstraal & Heynemann, 1969). The recent finding of possible *L. donovani* infections in dogs and some other animals (Mukhtar *et al.*, 2000; Dereure *et al.*, 2000; Elnaiem *et al.*, 2001) prompts the need for further investigations for a better understanding of the *Leishmania* species and their epidemiological status in Sudan. Many of the mentioned diagnostic tests are currently in use for the detection of *Leishmania* infection in animals.

**Over all Objectives**

- Characterise the *Leishmania* parasites present in the vertebrate reservoir(s) by specific markers.
- Disseminate the conclusions of the project into current or developing control programmes for visceral leishmaniasis in Sudan.

**Specific objectives**

1-To assess the success of infection with *Leishmania donovani* to Sudanese indigenous dogs (*Canis familiaris*).

2- To investigate if dogs are reservoir/hosts for *Leishmania donovani* and play a role of Leishmaniasis transmission.

3- To answer the following important questions:

(i) Can this infection progress to the CVL?
(ii) In case of progression of the disease, can we use the parameters and features as standard markers for canine reservoir host survey?
CHAPTER TWO

LITERATURE REVIEW

2.1 The leishmaniasis

Leishmaniasis is an important group of diseases affecting human and animals which are caused by various species of parasites of the genus *leishmania* Family: Trypanosomatidae, Order: kinetoplastida (Lainson & Shaw, 1987; Slapendel & Green, 1990). Leishmaniasis forced itself upon medical attention as an increasingly significant problem over the last decade. Because of its importance, leishmaniasis is considered as one of the six diseases selected by WHO for its special programme for research and training in tropical diseases (WHO, 1984). It ranks only second to malaria among human protozoan diseases (Chang & Bary, 1995). Leishmaniasis is endemic in 88 countries on the five continents (except Oceania), with a total of 350 million people are at risk and 12 million afflicted world-wide. There are 1-1.5 million new cases of CL and 500,000 of VL per year (Desjeux, 1996). Leishmaniasis is not a single disease but a variety of syndromes that differs remarkably with one to another.

2.2 The genus *Leishmania*

*Leishmania* parasites are haemo-flagellate protozoan. They are obligate intracellular parasites that are transmitted to the mammalian host by the bites of infected sandflies. On the basis of development in the sandflies, the genus
Leishmania has been divided into two subgenera. Development of organisms belonging to the subgenus Leishmania is restricted to the anterior portion of the alimentary tract of the Phlebotomus sandflies (suprapylyarian development), whereas organisms belonging to the subgenus Viannia develop in the midgut and hindgut of the sandflies Lutzomyia spp. (peripylyarian development). Viannia contains the complex of L. brasiliensis (L. brasiliensis, L. guyanensis, L. panamensis), L. mexicana, L. amazonensis and L. panamensis. The subgenus Leishmania contains the complex L. donovani (L. infantum, L. donovani, L. chagashi), L. major, L. tropica and L. aethiopica. There are in total at least 30 species, of which 12 named and several unnamed which infect man (Lainson & Shaw, 1987). Characterization was originally attempted on geographical and clinical grounds, and later on morphology, culture characteristics, biotopes and life cycles, antibody responses to particular antigens and lately on genetic analysis.

2.3. Life Cycle of Leishmania

Leishmania parasites are transmitted between long-living (humans, canines) vertebrate hosts by short-living Phlebotominae insects or sandflies (Lewis 1974; Lane 1993). They have a cycle of development in the vertebrate host and one in the insect, figure (1). Sandflies ingest amastigotes while sucking blood from the vertebrate host. The amastigotes transform into promastigotes in the alimentary tract of the sandflies (Lane, 1993). After 4-7 days they migrate to the foregut where, they develop into infective metacyclic forms. As the female sandfly has its blood meal, necessary before laying on of eggs, the mouthparts of the insect tear tissue and create a tiny pool of blood from
which they feed and into which metacyclic promastigotes are deposited. Metacyclic promastigotes are inoculated with sandfly saliva, which increases infectivity (Titus et al., 1988). The host cells are mononucleated cells e.g. macrophages. It is not precisely known how promastigotes enter the macrophages. In the macrophages they transform into amastigotes where they multiply and then they are deposited in different parts of the body (e.g: spleen, liver, bone marrow). Each *Leishmania* species has its own biotope with its own geographical distribution zone and complex of parasite, reservoir and vector and their particular intimate relationship within this setting. In Table (1) a summary of such relationships is presented.

**Table (1): Major *Leishmania* species that are of interest to the public health, their reservoir, vector and geographical distribution according to WHO (1996).**

<table>
<thead>
<tr>
<th><em>Leishmania</em> species</th>
<th>Vector</th>
<th>Reservoir</th>
<th>Geographical distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Old World</strong></td>
<td></td>
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</tbody>
</table>
| *L. infantum*       | *Phlebotomus perniciosus,*  
*P. ariasi*        | Dogs, foxes, jackals | Mediterranean basin,  
Middle east, China,  
Central Asia |
| *L. donovani*       | *P. argentipes*       | Humans | North-east India,  
Bangladesh, Burma |
| *L. donovani*       | *P. orientalis,*  
*P. martini*       | Rodents in Sudan,  
canines, humans,  
gerbils | Sudan, Kenya, Horn of  
Africa |
| *L. major*          | *P. papatasi,*  
*P. duboscqi*       | Gerbils  
(*Rombomys,  
Meriones*), Rodents | Middle East, North India,  
Pakistan, North Africa,  
Central Asia, Sub-Saharan |
Leishmaniasis is normally zoonoses infecting wild animals like rodents, edentates and canines. In each transmission cycle there is a restricted number of primary reservoir hosts that maintain the cycle. Sometimes there are

<table>
<thead>
<tr>
<th>Leishmania species</th>
<th>Reservoir Hosts</th>
<th>Geographical Distribution</th>
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</thead>
<tbody>
<tr>
<td>L. tropica</td>
<td>P. sergenti</td>
<td>Humans</td>
</tr>
<tr>
<td></td>
<td>(Arvicanthus, Tatera)</td>
<td>savannah, Sudan</td>
</tr>
<tr>
<td>L. aethiopica</td>
<td>P. longipes</td>
<td>Hyraxes</td>
</tr>
<tr>
<td></td>
<td>P. Pedifer</td>
<td>Highlands of Kenya, Ethiopia</td>
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<tr>
<td>New World</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. chagasi</td>
<td>Lutzomyia longipalpis</td>
<td>Foxes, Dogs, opossums</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Central America, Northern South America (Brazil, Venezuela, Yucatan, Belize, Guatemala)</td>
</tr>
<tr>
<td>L. brasiliensis</td>
<td>Lutzomyia spp., Psychodopygus wellcomei</td>
<td>Forest rodents, peridomestic animals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tropical forests of South and Central America</td>
</tr>
<tr>
<td>L. guyanensis</td>
<td>Lu. umbratilis</td>
<td>Sloths (Choleopus), arboreal anteaters (Tamandua)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guyana, Surinam, Brazil</td>
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<tr>
<td>L. panamensis</td>
<td>Lu. Trapidoi</td>
<td>Sloths (Choleopus)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Panama, Costa Rica, Colombia</td>
</tr>
<tr>
<td>L. mexicana</td>
<td>Lu. olmeca</td>
<td>Forest rodents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yucatan, Belize, Guatemala</td>
</tr>
<tr>
<td>L. amazonensis</td>
<td>Lu. flaviscutellata</td>
<td>Forest rodents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tropical forests of South America</td>
</tr>
<tr>
<td>L. peruviana.</td>
<td>Lutzomyia spp</td>
<td>Dogs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>West Andes of Peru, Argentine highlands</td>
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</table>


secondary hosts as well, that extend the cycle like the dogs in *L. chagasi* and accidental ones that are not important from the view point of maintaining the cycle like man in the case of *L. major*. It has been suggested that acute human cases of VL in Africa (not in Europe) are also serving as reservoirs. Nevertheless, new epidemiological results on HIV-*Leishmania* co-infected patients in the Mediterranean area and the post-kala-azar dermal leishmaniasis (PKDL) in India and Sudan (WHO, 2000), are overthoroughing such suggestions. PKDL is a condition by which man serves is a reservoir for *L. donovani* (Osman *et al.*, 1998). In the Old World sandfly vectors belong to the genus *Phlebotomus* and in the New World to the genera *Lutzomyia* and *Psychodopygus*. The breeding sites of many species are unknown. Breeding sites, flying habits, feeding habits, degree of anthropophilia or zoophilia (Lainson, 1983), efficiency of transmission (Dye, 1992), life span and biting during the night, are some of the important determinants of infection, and thus of control measures of the disease as well (Lewis *et al.*, 1987). When the reservoir and the vector share the same habitat like *P. papatasi* and the gerbil, then the risk of human infection becomes high (*L. major*). Where the habitats are separate but partly overlapping, the risk becomes relatively less, like in the case of *Lu. umbratilis* and *Lu. guyanensis* (Ashford *et al.*, 1987). Man and/or dog are usually infected by the bite of an infected sandfly. Visceral Leishmaniasis has been transmitted by sharing needles & by drug abusers (Le Fichoux *et al.*, 1999), sexual intercourse, accidental or deliberate inoculation in the laboratory, or congenitally (Sarman, 2006). Cutaneous leishmaniasis (CL) has been reported to be transmitted by deliberate scarification as a form of immunization (Gunders, 1987) and through suckling (Marsden *et al.*, 1985).
**Figure (1):** The life cycle of *Leishmania donovani infantum*

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2.4. Clinical spectrum

There are several different forms of leishmaniasis. The most common forms are cutaneous leishmaniasis, which causes skin sores, and visceral leishmaniasis, which affects some of the internal organs of the body (for example, spleen, liver, and bone marrow).

2.4.1. Cutaneous leishmaniasis (CL)

It is known as 'little sister' in some countries that the disease is so common that it is become a part of the family. In the Old World it is known as oriental sore. It produces skin lesions, sometimes as many as 200 on the face, arms and legs, causing serious disability and permanent scars (WHO, 1990). In the Old World it is caused by *Leishmania major*, *Leishmania tropica* and *Leishmania aethiopica*. In the New World CL is caused by *Leishmania mexicana* and *Leishmania braziliensis* complexes. Some *Leishmania infantum* and *Leishmania donovani* strains can also cause lesions. Ninety percent of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually world-wide (WHO, 1996). *Leishmania major* usually produces self-healing lesions, on the other hand, *Leishmania tropica* is usually more chronic, and its most severe form, recidivans leishmaniasis, is very difficult to treat. In the New World, *Leishmania mexicana* usually produces relatively benign lesions but some locations such as the ear's pinna are very difficult to treat in general (Desjeux, 1996).
2.4.2. Post kala-azar dermal leishmaniasis (PKDL)

It is a dermatropic form of leishmaniasis developed by part of the ex-VL patients (WHO, 1990), but there are cases without any previous known history of VL (El-Hassan et al., 1992). The disease is characterized by the development of macules, papules and nodules, which first appear around the mouth; those which do not heal spontaneously become denser and spread over the entire body (Berman, 1997). The interval between the end of treatment of VL and the onset of PKDL is variable: PKDL may appear during or directly after treatment (Zijlstra et al., 1995) to up to 2 years post treatment (Zijlstra et al., 1991). PKDL patients may be important sources of infection in VL transmission (Addy & Nandy, 1992; WHO, 1990).

2.4.3. Diffuse cutaneous leishmaniasis (DCL)

It is less common, chronic in evolution and especially difficult to treat. It produces lesions resembling leprosy, which do not heal spontaneously, due to deficiency of the immune response. DCL is due to Leishmania aethiopica and Leishmania amazonensis (Desjeux, 1996; WHO, 1996).

2.4.4. Mucocutaneous leishmaniasis (MCL)

Also called 'espundia', it produces disfiguring lesions to the face, destroying the mucous membranes of the nose, mouth and throat (Desjeux, 1996; WHO, 1996). It is mostly related to Leishmania species of the New World such as L. braziliensis, L. panamensis and L. guyanensis, but mucosal lesions have been reported in the Old World due to L. donovani, L. major and L. infantum in immunosupresed patients (Desjeux, 1996).
2.4.5. Visceral leishmaniasis (VL)

Also known in Asia as 'black fever' or 'kala-azar' is the most severe form of the disease, the parasite invades internal organs (spleen, liver, bone marrow) and the consequences are usually with an almost 100% mortality rate if left untreated. It is characterized by irregular fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathy and anaemia. VL is caused by *L. donovani* on the Indian subcontinent and in East Africa, by *L. infantum* in the Mediterranean region and by *L. chagasi*, which is closely related to or not distinguished from *L. infantum* (Mauricio *et al*., 2000), in the New World mainly in Brazil, Peru and Paraguay (Berman, 1997). Most forms of the disease are transmissible only from animals (zoonosis), but some can be spread between humans. Visceral leishmaniasis, caused by geographic variants of the *Leishmania donovani* complex (*L. donovani, L.infantum, L.chagasi*), is a progressive wasting disease of dogs and humans that is often fatal if untreated (Baneth, 2006). Agents of the *L. donovani* complex occur in parts of Mediterranean Europe, the Middle East, Asia, Africa, and Central and South America (Baneth, 2006. Herwalt *et al*., 2006). In infections involving the *L. donovani* complex in the Mediterranean region (*L. infantum*) and in South America (*L. chagasi*), dogs are reservoirs for human infection. Parasites are usually transmitted between hosts by phlebotomine sandflies (*Lutzomyia* or *Phlebotomus* spp.) (Herwalt *et al*., 2006; Jeronimo *et al*., 2006). Direct quantitative relationships between prevalence of leishmaniasis in local dog populations and incidence of human disease have been reported. Infection in dogs may indicate human risk for leishmaniasis, especially in HIV-positive persons, in many areas (Gradoni *et al*., 1996); infected but
asymptomatic dogs can infect sandflies that feed on them, posing a risk to uninfected dogs and humans (Reithinger et al., 2002a).

2.5. Inhabitance at risk of Leishmaniasis

People of all ages are at risk for leishmaniasis if they live or travel where leishmaniasis is found. Leishmaniasis usually is more common in rural than urban areas; but it is found in the outskirts of some cities. The risk for leishmaniasis is highest from dusk to dawn because this is when sand flies are the most active. All it takes to get infected is to be bitten by infected sand fly. Adventure travelers, Peace Corps volunteers, missionaries, ornithologists (people who study birds), other people who do research outdoors at night, and soldiers are examples of people who may have an increased risk for leishmaniasis.

2.6. Epidemiology of Leishmaniasis

The epidemiology of leishmaniasis in a given area is directly dependent on the behaviour of the human and/or animal population in relation to the cycle of transmission. There is a variety of factors that influence the transmission of the disease. Some are the following (for review see Kettle, 1995 and Lane, 1993):

(a) Proximity of residence to sandfly breeding and resting sites.
(b) Type of housing.
(c) Occupation.
(d) Extent of exposure to sandfly bites.
(e) Natural resistance, genetic or acquired.
(f) Virulence of the parasite species.
(g) Zoonotic or anthroponotic reservoirs.
It seems that zoonotic reservoirs are particularly stable when wild uncontrolled populations (e.g. rodents) are involved. Up to now it seemed that humans are not a reliable agent because of death and treatments except of the chronic condition of PKDL. Nevertheless, recent reports about asymptomatic infections in healthy blood donors in France (le Fichoux et al., 1999) are adding a new parameter to the latter.

- The vectorial capacity, which is defined as the number of infective bites delivered per human per annum (Dye, 1992).

- Density, seasonality, longevity and flight range of sandfly populations.

- Anthropophilia or zoophilia of sandflies and degree of it.

Rarely, leishmaniasis is spread from a pregnant woman to her baby. Leishmaniasis also can be spread by blood transfusions or contaminated needles.

### 2.7. Distribution

The geographic distribution of leishmaniasis is cosmopolitan, figure (2) and (3). Thus *L. tropica* and *L. major*, causing Oriental sore are found in Russia, Indonesia, equatorial Africa, in the west and east of the Mediterranean (Italy, Spain, Greece, Bulgaria and Romania). For instance, *L. mexicana* is found in the southeast of México in Tabasco, Campeche and other states, Central America and some countries of South America like Venezuela. *Leishmania brasiliensis* and its subspecies cause mucocutaneous leishmaniasis or espundia localized in South and Central American countries and the southeast of Mexico, principally Tabasco and Quintana Roo.
*Leishmania peruviana* found in the Peruvian slopes of the Andes and Argentine highlands. *Leishmania donovani* with its subspecies and closely related ones like *L. infantum* are found in the Mediterranean basin, Africa, regions of Asia and in America as *L. donovani chagasi* localized mainly in Brasil, Venezuela, Colombia, El Salvador, Guatemala and in Mexico in the Balsas valley.

**Figure (2):** Distribution of Old World and New World cutaneous leishmaniasis

**Figure (3):** Distribution of Old World and New World visceral leishmaniasis

Data source: WHO/CSR/EDC-UNAIDS  
2.8. Leishmaniasis in the Sudan

Sudan is considered as one of the most important areas of leishmaniasis in the world where sharp epidemics involving thousands of people with many deaths were recorded. All forms of leishmaniasis i.e. CL, MCL, VL and PKDL occur in Sudan. This group of diseases causes serious economic loss in the country, both in terms of the disability of affected individuals and in the cost of treatment, especially as most of those with leishmaniasis are on low incomes and live in rural areas (cited from El Tai. Nahla. O, 2003). Visceral leishmaniasis (VL) is the one of the most important endemic diseases in the country and is known to occur in the Sudan since 1904 when Neave (1904) described the first patient in the country. The main endemic area is in the eastern part of the country, from the banks of the White Nile in the West to the Ethiopian border in the East, and from Kassala in the North towards Malakal in the South, figure (4). Other smaller foci have also been described in Kapoeta in Equatoria and parts of Kordofan and Darfur provinces. Occasional severe outbreaks occur, like the one in the southern Fung in Blue Nile province in 1956-1960, which caused thousands of death (Sati, 1958). An outbreak of kala-azar was reported in Khartoum among displaced people (de Beer et al, 1990). Also, epidemics have occurred in recent years in war zones of southern Sudan where about 100,000 people died of leishmaniasis since 1984 (Seaman et al, 1996). Recently (Robert et al., 2000) reported that more than 10% of the population in southern Sudan died from visceral leishmaniasis over the past 5 years. Zeese and Frank (1987) estimated that of the total number of 1300 patients reported annually in the Sudan, more than 75% were treated in the hospitals of Gedaref and
Hawata, a small rural town ~100km south to Gedaref, situated along the Rahad River.

Figure (4): Endemic areas of different forms of Leishmaniasis in Sudan. From Osman, (1997)
2.9. Diagnosis of leishmaniasis

Routine diagnosis of VL relies on a combination of the following methods:

(i) The clinical suspicion of VL may be confirmed directly by (a) microscopic detection of leishmanial amastigotes in stained smears of lymphnode, bone marrow or splenic aspirates (b) the culture of the parasite (Weiss, 1995; Osman et al., 1997). (ii) Immunological tests for the detection of anti-leishmanial antibodies and leishmanial antigens and (iii) molecular techniques including PCR.

2.9.1. Parasitological diagnosis

The material obtained can be used to prepare a smear that will be stained with Giemsa, Wright's or Leishman's stain, or inoculate culture media for the isolation and the culture of the parasite (Sells et al., 1981).

2.9.1.1. Microscopy

The routine diagnosis of leishmaniasis relies on the microscopic detection of Leishmania amastigotes in Geimsa stained aspirate materil from lymph nodes, bone marrow, spleen or liver, in slit skin smear or in peripheral blood. The method is relatively simple and cheap, but has limited sensitivity (Weiss, 1995; Osman et al., 1997) and there no possibility to distingueish between Leishmania amastigotes belonging to the different species (Weiss, 1995).

Microscopy on lymph node aspirates is the most commonly used procedure for the confirmation of diagnosis of VL in Sudan. The method had been recommended as a safe procedure (Siddig et al., 1988), but sensitivity is
reported to be variable ranging from 58.3% (Zijlstra et al., 1992) to 100% (Kirk and Sati, 1940). Intermediate sensitivities of 78% (Siddig et al., 1988) and 64.5% were also reported, suggesting that lymph node aspirate was equal in sensitivity to bone marrow aspiration but only 2/3 as sensitive as splenic aspirate (Siddig et al., 1988). In the past *Leishmania* parasites were easily demonstrated in the peripheral blood in cases of India VL but not so more recently. Elsewhere, particularly in Sudan, the percentage of parasite-positive peripheral blood samples has been very low, ranging from 0-3% (Archibald and Mansour, 1937; Henderson, 1937; Kirk and Sati, 1940); with the exception of one study dating from the beginning of this century when 86% of the blood samples were found in the veinous blood of 7 of 20 confirmed VL patients.

### 2.9.1.2. Culture

*Leishmania* parasites can be cultured in a range of media where they grow as promastigotes: slender spindle like organisms with a flagellum. Commonly used culturing media include: (i) semi-solid (Novy-McNeal-Nicolle, NNN-medium) and liquid media with blood components as an essential factor; (ii) insect culture media; and (iii) semi-defined liquid tissue culture media such as RPMI-1640, Mem and TC199. These different media vary in their efficacy to transform amastigotes obtained from smears into promastigotes and in the growth rate of the promastigotes.

Other possibilities of culturing are animal inoculation, especially hamster’s inoculation. In these methods, 0.1 ml of patient material (usually aspirate or biopsy) is inoculated subcutaneously into the tip of the nose of a hamster
which is highly susceptible for infection with *Leishmania*. When nasal inflammation is noted during bi-weekly examination, dermal scarpings from the nose are prepared and searched for amastigotes (Weigle *et al*., 1987). In the absence of nasal inflammation, the hamster will be killed 6 weeks later and materials from the spleen of the animal are prepared for subsequent microscopic examination and/or culturing in appropriate medium (El-Hassan *et al*., 1995). Bush baby *Galago senegalensis* has also been described as an experimental host for Leishmaniasis (Sati, 1963).

### 2.9.2. Molecular diagnosis of leishmaniasis

PCR is a technique which allows the sensitive, specific and fast detection of minute amounts of pathogen DNA, even in the presence of excess amounts of host DNA. PCR is based on the amplification of a known, specific sequence using oligonucleotide primers (typically 20 mers) which specifically bind to the DNA flanking the region of interest. The target sequence is amplified using a heat-stable DNA polymerase isolated from *Thermos aquaticus* (Saiki *et al*., 1988). First the double stranded (ds) target DNA is denatured at 94°C after which the primers are allowed to anneal to the single stranded (ss) DNA at asset temperature which has to be determined for each primer set (typically 50-65). The annealing step is followed by primer extension by *Taq* polymerase at 72°C. Thus, in each amplification cycle one ds DNA target molecules gives rise to two ds DNA copies, which can then serve as target sequences for the next cycle. This means that, in theory, one double strand DNA molecule before amplification gives rise to approximately $10^9$ copies after 35 amplification cycles. PCR products can be visualized with strand
molecular biological methods such as gelelectrophoresis and Southren blotting.

One of the major complications of the use of PCR is the detection of false-positive due to “sample contamination”, which arises from the carry-over of amplicons. This can be prevented by: (i) a stricted separation of areas for:

(a) Sample preparation. (b) PCR reaction mixture preparation and (c) PCR amplification and detection (Kwok and Higuchi, 1989).

(ii) Using the dUTP/dUNG system described by Longo et al., (1990). Over the years a number of different PCR assays have been developed for the detection of *Leishmania* DNA in clinical samples.

2.10. Canine Visceral Leishmaniasis (CVL)

Dogs are considered as the most important vertebrate reservoir of the Leishmaniasis. The incubation period of the disease may range from 30 days to four years, so the appearance of clinical signs can not be considered seasonal (Liste and Gascon, 1995). Canine leishmaniasis (CVL) is a severe systemic disease with hair loss, skin lesions, epistaxis, anemia, wasting, swollen limbs and joints, lameness, renal failure, lymphadenopathy, ocular lesions, and diarrhea (Slappendel and Teske, 1999). Canine leishmaniasis (CVL) caused by the protozoan parasite *Leishmania infantum*, is a common infectious disease in the countries of the Mediterranean basin and Portugal. CVL is a disease with variable clinical picture, where almost every organ system is involved (Alvar et al., 2004; Baneth, 2006). In the dog, clinical features are complex, with different clinical signs depending on individual
variation, type of *Leishmania* and phase of the disease (Abranches *et al.*, 1991). In particular, it may include progressive loss of body weight, cutaneous, ocular, and musculoskeletal signs, renal and liver disease, peripheral lymphadenomegaly, hepatosplenomegaly, and epistaxis. Agents of the *L. donovani* complex occur in parts of Mediterranean Europe, the Middle East, Asia, Africa, and Central and South America (Alvar *et al.*, 2004; Baneth, 2006; Koutinas *et al.*, 1999). In infections involving the *L. donovani* complex in the Mediterranean region (*L. infantum*) and in South America (*L. chagasi*), dogs are reservoirs for human infection. Canine VL is regarded as both an important veterinary problem and a problem concerning human health, as the dog is the main reservoir of *L. infantum* and *L. chagasi*. An increase in both VL and canine VL cases has been reported in most Mediterranean countries in recent years (WHO, 1990). Studies in European foci have shown that the prevalence of CVL varies from 1 to 37% (Alvar *et al.*, 2004. Bettini and Gradoni, 1996). In addition, infections are now frequently reported as import cases in northern Europe (Slappendel and Teske, 1999).

### 2.10.1. Geographical distribution

The geographical distribution of different *Leishmania* species is directly dependent on the local presence of the respective specific sandfly vectors. *Leishmania infantum*, the cause of canine leishmaniasis in the “old world” is transmitted by *Phlebotomus spp.* that lives mainly in the Mediterranean, the Middle East and North Africa. More specifically; foci of the disease exist in Albania, Algeria, Bosnia-Herzegovina, Chad, Croatia, Cyprus, Egypt, France, Greece, Islamic Republic of Iran, Iraq, Israel, Italy, Jordan, Lebanon,
Libyan Arab Jamahiriya, Malta, Morocco, Portugal, Saudi Arabia, Spain, Syrian Arab Republic, Tunisia, Turkey and Yemen. Besides these regions, foci of low endemicity are found in Azerbaijan, Georgia, Kazakhstan, Turkmenia and some provinces of China.

Between and even within all of these countries, the prevalence of leishmaniasis varies considerably; seroprevalence in domestic dogs in the Mediterranean basin is reported to range from 1.4% to 48.4% but is on average around 10% (Gradoni, 1999; WHO, 1990). In the “new world” canine leishmaniasis is caused by *L. chagasi*. This species is however indistinguishable from *L. infantum* and is considered to be the same parasite on the basis of genetic data (Mauricio *et al.*, 1999). In the new world the parasite is transmitted by a different vector species: sandflies of the genus *Lutzomyia*. Foci are present in Bolivia, Brazil, Colombia and Venezuela and possibly also in Argentina, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama and Paraguay. Seroprevalence in dogs ranges from 24% to 67% in high endemic clusters (Miles *et al.*, 1999; WHO, 1990).

### 2.10.2. Prepatent period

Of the low numbers (1 to 100) of metacyclic promastigotes that are delivered with the bite of a sandfly only few survive and transform into intracellular amastigotes (Warburg *et al.*, 1986). Therefore, it takes some time for the parasites to multiply and spread from the site of infection. As host factors influence parasite spread and multiplication, it may take between 2 months and many years for symptoms of disease to become apparent (Adler *et al.*, 1934; Slappendel, 1988). The long and variable prepatent period complicates
epidemiological and intervention studies in the field as well as experimental infection models in the laboratory.

2.10.3. Clinical disease spectrum

Following acquisition of infection, dogs can remain asymptomatic for variable periods or never develop clinical manifestations. Clinical manifestations can include chronic wasting, conjunctivitis, ocular signs (anterior uveitis, retinitis), facial alopecia, severe muscle atrophy, lymphadenopathy, polyarthritis, and protein-losing nephropathy, which may lead to renal failure. Infection may result in severe systemic disease with hair loss, skin lesions, epistaxis, anemia, wasting, swollen limbs and joints, lameness, renal failure, lymphadenopathy, ocular lesions, and diarrhea. In CVL 90% of symptomatic dogs have cutaneous manifestations (Slappendel, 1998). Unlike human CL in which the parasite is restricted to the skin, in CVL the cutaneous lesion results from dissemination of the disease from the internal organs (Ferrer, 1999). Infected dogs may remain asymptomatic for variable periods or never develop clinical manifestations.

Dogs suffering from leishmaniasis show skin problems, weight loss and/or decreased activity. Upon examination by the veterinarian, lymphadenopathy is found in 90% of cases (Ciaramella et al., 1997; Slappendel, 1988). Skin disease is also frequently found (Slappendel, 1988) and appears as dry exfoliative dermatitis, ulcers, diffuse alopecia. Less frequent symptoms include weight loss, anorexia, abnormal locomotion, conjunctivitis, splenomegaly and onychogryphosis. Other symptoms that may be present include (masticatory) muscle atrophy, diarrhea, epistaxis, ocular involvement
(keratitis, panophthalmitis), and hyperthermia. In the final stages of the disease, signs of renal failure may develop, such as anorexia, polyuria/polydipsia and vomiting.

### 2.10.4. Diagnosis of Canine leishmaniasis

Diagnosis of visceral leishmaniasis in dogs is based on positive specific antibody assay with confirmation by demonstration of the parasites (amastigote forms) on touch prep stained (Wright-Giemsa) slides or in cultures of tissue aspirates or biopsy specimens of the spleen, liver, bone marrow, or lymph nodes.

Diagnostic antibody tests include the indirect fluorescent antibody assay (IFA), direct agglutination assay, and enzyme immunoassay (EIA). These tests vary in sensitivity and specificity, and although they verify presence of antibody, they do not prove or rule out active infection. Most of these assays give false-positive reactions with sera of dogs infected by *Trypanosoma sp*, another protozoan that sometimes infects dogs. (Zandra *et al.*, 2000).

#### 2.10.4.1. Clinical diagnosis

Although individual symptoms of leishmaniasis are mostly non-specific, a dog exhibiting a combination of the above mentioned signs is likely to be adequately diagnosed by those that are familiar with the disease. However, depending on the symptoms that are present, leishmaniasis may be difficult to distinguish from infectious diseases like ehrlichiosis, hepatozoonosis or babesiosis. Co-infections with these parasites may also occur. Immune-mediated diseases like polyarthritis, hemolytic anemia or systemic lupus
erythematous, skin diseases like demodicosisor dermatophytosis, endocrine disorders and malignancies like myelo-and lymphoproliferative diseases can also cause similar clinical manifestations.

2.10.4.2. Laboratory diagnosis

Due to the rather variable clinical presentation of Leishmaniasis, any clinical suspicion of disease should be confirmed in the laboratory. These tests are either based on identification of the parasite or on serology. The detection of parasites is the ‘gold standard’ for diagnosis; its sensitivity however is not high.

2.10.4.3. Parasitological methods

Parasites in clinical samples can be demonstrated either directly or after multiplication in *vitro* culture. Microscopic examination of Giemsa-stained smears prepared from bone marrow or lymph node aspirates is a classical method with high specificity but low sensitivity, approximately 60%.

Examination of histological samples taken from skin, lymph node, spleen or liver is also of use; the histopathological picture however is not specific for leishmaniasis and only the presence of amastigotes can confirm the diagnosis. As the number of amastigotes in tissue samples is often very low, diagnosis can be difficult. The sensitivity of histopathology can be increased by applying immuno-histochemistry using specific antibodies to detect the presence of *Leishmania* in tissue samples (Ferrer *et al.*, 1988; Sells *et al.*, 1981). Samples of bone marrow, lymph node and/or spleen can be cultured in *vitro* at allowing the transformation of amastigotes to promastigotes and
subsequent multiplication of the latter. As multiplication of parasites occurs in culture, this enhances the chances of parasite detection in clinical samples. Furthermore the method is 100% specific as the identity of promastigotes in culture cannot be mistaken. On the other hand, many factors can influence parasite growth and depending on the circumstances cultures may falsely be scored negative. Factors influencing the success of culture methods are: the type of culture medium, the length of the incubation period, the amount of sample inoculated in a given amount of medium and the number and type of samples taken. Still, when optimal conditions are met, the sensitivity of parasite culture may approach 100% (Mathis et al., 1995).

2.10.4.4. Molecular diagnosis

In more recent years, the use of PCR for parasite detection has gained much attention. Several different primer combinations have been tested, some targeting genomic DNA sequences, others the kinetoplast of which several thousand copies are present in each parasite.

The latter type of PCR test is generally found to be the more sensitive (factor 500 to 5000) (Lachaud et al., 2002a; Lachaud et al., 2002b). Many different types of samples have been used for these tests including bone marrow, lymph node, spleen, skin, peripheral blood and even conjunctival swabs (Ashford et al., 1995; Barrouin-melo et al., 2004; Reale et al., 1999). In general the specificity as well as sensitivity of the tests is estimated to be close to 100%. In the field, evaluation of PCR tests is hampered by the absence of a real “gold standard”. Due to the occurrence of extremely low parasite burdens, non-specific clinical signs and late development of
serological responses it is often not clear whether a dog should be designated *Leishmania* positive and it therefore remains uncertain whether a PCR result is true or false positive. In experimental infection systems, the parasite burden is widely used as a parameter of disease severity. The use of Real-Time PCR for quantification of *Leishmania* has recently been reported (Bretagne *et al*., 2001; Schulz *et al*., 2003).

### 2.10.4.5. Serological methods

Because of the ease of serum sampling and testing, serological methods are widely used for clinical as well as epidemiological purposes. Many different methods have been developed, differing in the type of antigen and/or the detection system used. Antigen may consist of whole killed parasites, total soluble antigen or purified natural or recombinant *Leishmania* antigens. Detection systems used include Direct Agglutination Test (DAT), Immune Fluorescence Assay (IFA) and several modifications of the Enzyme Linked ImmunoSorbent Assay (ELISA). Evaluation revealed relatively high sensitivity (99.5-100%) and specificity (95-100%) without great differences between the different serological assays tested (Harith *et al*., 1989; Mancianti *et al*., 1995; Rachamim *et al*., 1991; Vercammen *et al*., 1997).

### 2.11. Laboratory models for *Leishmania infantum* infection

Models for visceral leishmaniasis are problematic. Although mice can be infected with *L. infantum*, an immune response develops and clears the parasite a few weeks after challenge. This is in contrast to the lethal uncontrolled dissemination of the parasite seen in dogs. An alternative model
species is the hamster, which is very susceptible to *L. infantum*. The almost complete lack of immunological tools for hamsters is a serious disadvantage and it is unclear to what extent development of immune responses in hamsters and dogs are comparable. For canine leishmaniasis research, the dog is undoubtedly the best model species. Besides this, the dog is also regarded to be a good model for visceral leishmaniasis in humans. As research in the human field relies on artificial parasite-host models, this area would benefit greatly from developments in the veterinary field. Disadvantages however include cost, limited immunological tools, long incubation period and ethical considerations. These are probably the most important reasons for the limited number of experiments that have been performed in dogs.
2.12. Natural history of canine leishmaniasis

Fig. 5 Natural history of canine leishmaniasis. Different phenotypes of dogs which could occur following a *Leishmania*-infected sandfly bite are indicated: healthy non-infected (green), symptomatic (red) and asymptomatic (blue) dogs. The sandfly symbol indicates situations in which the infected dog is capable of transmitting *Leishmania* to the insect vector, as proven by xenodiagnosis. The factors affecting each step are not clear; hence, it is not possible to determine why infected dogs remain asymptomatic, why ill dogs can heal spontaneously or suffer clinical reactivation after treatment.
2.13. Experimental infection of dogs

A limited number of publications regarding the experimental infection of dogs with *L. infantum* are available. Small numbers of dogs were used in these experiments (24 dogs divided to 2 groups) and as a consequence it is difficult to compare them and draw general conclusions about inoculation route, dose or parasite stage. The intravenous route is most commonly used, compared to the intradermal route the former appears more likely to result in the development of clinical disease (Paranhos-Silva *et al.*, 2003; Santos-Gomes *et al.*, 2000). It is generally accepted that the most reproducible and virulent infections are obtained by injection of amastigotes (Abranches *et al.*, 1991; Campino *et al.*, 2000; Hommel *et al.*, 1995). The dose of parasites varies significantly between experiments, ranging from $5 \cdot 10^5$ to $1 \cdot 10^{10}$ per dog (Campino *et al.*, 2000; Nieto *et al.*, 1999). Good results with regard to parasite detection, development of disease and reproducibility were obtained with a model in which dogs are inoculated intravenously with $4 \times 10^8$ stationary phase culture promastigotes (Riera *et al.*, 1999; Valladares *et al.*, 1998). Diagnosis of experimental CVL is, in general, mainly relying on serology and parasitology. Although differences in humoral response between experimentally and naturally infected dogs have been reported by some authors (Leandro *et al.*, 2001; Rhalem *et al.*, 1999), this was contradicted by others (Martinez-Moreno, 1995). The outcome to the detection of parasites is very variable between the different experiments reported. This may be caused by differences in infection dose, injection route or parasite strain that can all influence development of the parasite burden. However, differences in sensitivity of the applied parasite detection methods
could also be an important factor. Although most of the above mentioned challenges result in infection of dogs, there are several disadvantages associated with the use of such models in vaccination-challenge experiment.
CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

This study was carried out in a non-endemic area of Visceral Leishmaniasis (Khartoum state) (15° 37’ 59” N, 32° 31’ 59” E) figure (6), twenty four Puppies in age three to six months were collected randomly from different areas at Khartoum State (i.e. Omdurman, Khartoum North & Khartoum), during the period 30 May to 17 October 2006. The dogs were collected manually.

Figure (6): The satellite image shows the study Area Khatroum state.

The Circle shows the Police Dogs’ Directorate, where the study held.
3.2. Study site

This study was carried out from Dec 2006 to Dec 2008, after a vigorous survey to explore the suitable place to keep the dogs at Khartoum State, the Police Dogs’ Directorate (plates (1)), has been selected and recommended by Dr/Sima Rafati (WHO visitor to RCS project, Dec 2005).

Plates (1): The cages where dogs were kept at Police Dogs’ Directorate (Khartoum State).

The area was free of *Leishmania* vector, this was based on study carried out in Police Dogs’ Directorate by El Fatih, H B.Sc student, on (Feb, March, and May 2007). The buildings of the administration and the area of Nile Street behind it were included in the study (El Fatih, H.,2007).
3.3. The parasite

Dogs were inoculated with *Leishmania donovani* (MHOM/SD/81/MW81). This strain was isolated from a Post Kala-azar Dermal Leishmanisis (PKDL) patient, cultured in NNN medium and incubated at 26°C. Promastigotes were collected from the stationary phase of a subculture. The promastigotes that were used in this study, were obtained kindly from the Institute of Endemic Diseases, University of Khartoum.

Table 2: Characterised human *Leishmania* strain used in study:

<table>
<thead>
<tr>
<th>WHO Code based on isoenzyme (analysis)</th>
<th>Taxon Origin</th>
<th>Patient’s clinical picture</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHOM/SD/81/MW81</td>
<td><em>L. donovani</em></td>
<td>Sudan</td>
</tr>
</tbody>
</table>

3.4. Experimental set-up

Twenty four out-breed dogs, of both sexes, were housed in the Police Dog’s Directorate facility under stable conditions throughout the experiment. The animals were housed individually in conventional kennels and fed a standard commercial diet (Canex; Hope Farms, Police Dog’s Directorate) throughout the experiment. Before inoculation, all collected dogs were treated with antihelminthics, vaccinated against leptospirosis, parvovirosis, hepatitis, distemper (DHPPi), and rabies, plate (2). Physical examination was obtained
by veterinary personnel. Selected Dogs has been tested to be free from infection with *Leishmania donovani*. Dogs were treated with antihelminthes as well as tick control process (twice a week), using basin (Dipping) method and spray method in addition to fogging of the cages.

Plate (2-A)

Plate (2-B)  Plate (2-C)

**Plate (2):** Vaccination with DHPPi

A- 1 ml of DHPPi vaccine.

B- The ice container where vaccine was storaged.

C- Dogs’ vaccination by DHPPi.
3.5. Inoculation

Seventeen dogs at age six months were inoculated intravenously, with a dose of approximately $4 \times 10^8$ promastigotes in 1 ml of isotonic PBS, plate (3). The remaining seven dogs were used as a control. This injection was placed in the left hind leg (in the region drained by the popliteal lymph node). According to protocols for the animal experiments were evaluated by an ethical committee (Cited from Jaquelin Poot, 2006). The experiments were performed in accordance with regulations for the use of experimental animals.

Plate (3): Dose with concentration ($4 \times 10^8$ promastigotes) in 1 ml of isotonic PBS used in present study for inoculation.
3.6. Sample collection

The prephiral blood sample was taken each month from the vein, plate (4). The blood was spotted on filter paper (Watman no.3), plate (5) for the DNA extraction to detect the *Leishmania* parasite by molecular method. Also blood was collected in EDTA tubes to study the blood haematological parameters; and was smeared on a slide to detect the *Leishmania* parasite by a microscope technique. Other tissues (Lymphnode and bone marrow) were collected in eppendorf tube containing STE lysis buffer & the tissues biopsy were spotted on filter paper and cultured in NNN-media.

Plate (4): The blood sample collection from vein.

Plate (5): Blood spotted on filter paper Wattman no.3
3.7. Clinical examination and the body weight

During clinical examination the size of popliteal lymph nodes was established, general body condition was assessed (the body weight was recorded and claws were observed every month). Skin change was scored (0: normal, 1: slight, 2: severe (for alopecia, lesions, change of fur color and the hair loss). Obvious clinical abnormalities were recorded when present.

3.8. Blood Heamatology

The peripheral blood from vein was collected monthly in EDTA tubes to study the blood characters related to CVL, total blood cells count were done, and the Haemoglobin was estimated associated with Packed Cells Volume value to study the anaemic cases. The blood was studied by Sysmex machine model (CTH- 21), in El-mawda Hospital (El said Abd El Rahman Street-Khartoum State), for the first year of study (Dec 2006- Dec 2007) and Sysmex machine model (KX-21) in Modern Diagnosis Laboratory (Hospital Street- Khartoum State), till Nov 2008.

3.9. Parasitological studies

Parasitological examinations were carried out by popliteal lymph node tissue biopsy and preparations stained with Giemsa stain were examined by microscope. This material was also cultivated in NNN medium for parasite isolation.
3.10. DNA extraction

DNA was isolated as described by Meredith et al., (1993). Briefly, a paper punch was used to cut discs from the filter papers on which the spirates were stored, each paper being held between two sheets of clean paper while punched. After discs were cut for each sample, a clean sheet of paper was punched 10-12 times in order to prevent DNA cross-contamination. Two discs (together equivalent to about 15μl aspirate) were placed in 250 μl lysis buffer (as described by Sambrook, 1989) (50mM NaCl, 50mM Tri-HCL, (pH7.4), 10mM EDTA, 1%(V/V) Triton X-100 and 200μg proteinase K/ml], and incubated overnight at 56°C. The samples were then subjected to phenol/chloroform extraction, and redissolved in 50 μl TE buffer (10 mM Tris-HCl, 1mM EDTA; pH 7.5). Positive and negative controls were randomly added to check for contamination and inhibition.

3.11. Polymerase Chain Reaction (PCR)

Four primer pairs were used to detect the *Leishmania* parasite. Their characteristics are presented in Table (3). Through optimization of the PCR conditions was carried out as described previously for each the PCR methods. Using dog blood sample, the optimize conditions for each primer pairs are summarize in Table (3). For all reactions 2.5μl of 10x PCR buffer, 0.5 of deoxynucleotides triphosphate, 0.2 μl of *Taq* DNA polymerase (CinnaGen Co.) and 2 p/mol of each primer pairs were used in a total reaction volume 25 μl, including 5μl of DNA sample. The variable factors
included the MgCl₂ concentration, and the primer annealing temperature. The reactions were cycled in (TECHNE, TC-412) thermal cycler and in the following conditions were used: pre-incubated at 110°C for 4 minutes, followed by initial denaturation at 95°C for 5 mins and 35 cycles consist of denaturation at 94°C for 1 min, variable annealing temp according to the primer set (Table 3) for 1 min, and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 10 minutes. Each concentration for the sensitivity assays. Additionally, in each test, one internal positive control tube for the detection of PCR inhibition was included for each sample. Finally, one negative control tube that receives 5 µl of ddH₂O instead of DNA was included in each test to detect any carryover contamination.
**Table (3): Main characteristics of the four PCR methods used in the study**

<table>
<thead>
<tr>
<th>PCR DNA target</th>
<th>Pia1-Pia2</th>
<th>Pia3-Pia4</th>
<th>DB8-AJS3</th>
<th>RV1-RV2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repititive sequence of <em>Leishmania infantum</em> genome</td>
<td>Repititive sequence of <em>Leishmania infantum</em> genome</td>
<td>kDNA (minicycle 10,000 copies)</td>
<td>kDNA minicircle (10,000 copies)</td>
<td></td>
</tr>
<tr>
<td>Product size(bp)</td>
<td>100 bp</td>
<td>120 bp</td>
<td>809bp</td>
<td>145 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>700bp,400bp</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td><em>L. donovani</em> sensu lato</td>
<td><em>L. donovani</em> sensu lato</td>
<td><em>Leishmani sp</em></td>
<td><em>L. donovani</em> sensu lato</td>
</tr>
<tr>
<td>Primer sequences</td>
<td>5’ACGAGGTCAGCTCCACTCC3’</td>
<td>5’CGGCTTCGCACCATTGCGCGT3’</td>
<td>DB8: 5’CCAGTTTCCGCCCCCGAG3’</td>
<td>5’CTTTTCTGGTCCCCGGGGTAGG3’</td>
</tr>
<tr>
<td></td>
<td>5’CTGCAACGCCTGTGCTACG3’</td>
<td>5’ACATCCCTGCCACATACGC3’</td>
<td>AJS3: 5’GGGTTTGTGTTAAATAGGG3’</td>
<td>5’CCACCTGGCCTATAACACCA3’</td>
</tr>
<tr>
<td>MgCl₂ concn</td>
<td>3µl</td>
<td>1.5 µl</td>
<td>4 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>Annealing temp</td>
<td>64°C</td>
<td>63°C</td>
<td>54°C</td>
<td>59°C</td>
</tr>
</tbody>
</table>
3.12. Statistical analysis

Statistical analysis was performed using statistical package for social science (SPSS ver.13). RBCS, WBCS, platelets, and body weight were found not normally distributed, so the data were normalized and then Student T. test was used to compare the means of these parameters between control and infected dogs within different periods of study. The Data of hemoglobin and Packed Cell Volume (PCV) were not normally distributed and did not show normality after transformation (can not be normalized), so the nonparametric test Mann-Whitney U was used in comparisons the means of these parameters through the periods of experiment between the infected and control group.
CHAPTER FOUR

RESULTS

4.1. Incubation periods

In this study seventeen dogs were infected experimentally by the promastigotes of *Leishmania donovani* parasite from stationary phase culture, by a dose of concentration $4 \times 10^8$. The clinical signs of CVL were observed two months post infection, the parasitological results were presented three months post infection, and the *Leishmania donovani* DNA was traced in all 17 infected dogs 16 months post infection. It was a success of infection. The incubation periods (16 months) the duration was one.

4.2. Clinical signs

Most (88%) of the intravenously (i.v), inoculated dogs developed clinical signs of leishmaniasis during the course of the study, although the severity and duration of these clinical abnormalities varied between individual animals. The first signs of disease became apparent between 8 and 15 weeks post infection (p.i.) in 15 out of 17 of the i.v. inoculated dogs.

These signs were mild and mostly involved either (enlarged) lymph nodes or (decreased) body condition or both, and sometimes accompanied by mild conjunctivitis. More serious clinical symptoms became evident between 35 and 40 weeks post-infection in about half of the dogs (8 out of 17). Besides serious weight loss and lymphadenopathy, disorders of skin
and coat appeared, while diarrhea was noted on two occasions (once in dog 23 control group).

Nine dogs were oligosymptomatic (more than two signs), showing mild clinical signs from about 20 weeks p.i. until the end of the study.

Two dogs remained asymptomatic for the duration of the experiment. The occasional mild clinical signs in these dogs (dogs 8 and 16 (Males).

Rectal temperature was monitored for all dogs (17 infected, &7 non-infected as controls). Fever (>40°C) was not detected during (24 months), significant differences between the control and the infected groups were not found.

Infected dogs were grouped according to clinical status into two groups (symptomatic dog (15 dogs), and asymptomatic (two dogs). Symptomatic dogs showed most CVL sign, table (4).

In figure (6), the average clinical score of the experimental dogs was shown as:

Thirteen dogs out of seventeen of infected dogs (76.5%) had lymphdenomegaly two months post infection and seven dogs (41.2%) showed Splenomegaly seven months post infection.

Three infected dogs out of seventeen (17.65%) were losted their hair, plate (6). Three infected dogs (17.65%) change the colour of the fur, plate (7) six months post infection.
**Conjunctivitis:** only one dog (female) out of seventeen (5.9%) showed conjunctivitis manifestation, plate (8) shows this symptom as a sign of CVL nine months post infection.

**Abnormal skeleton (Bones problem):** two dogs out of seventeen (11.8%) had bone problem six months post infection and had abnormal locomotion, plate (9).

**Nail colour:** Some dogs had normally black nails, plate (10). Three dogs out of seventeen with white nail (17.6%) of infected dogs had colour change of their nails; plate (11) shows this sign three months post infection.

**Nail growth:** Seven dogs out of seventeen of infected dogs (41.18%) showed nails elongation four months post infection, plate (12) shows the normal length of dogs’ nails; plate (13) present the elongation and extention of nails of infected dogs.
Table (4): Characteristics of the Dog’s clinical signs and ratio of severity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Infected group</th>
<th>Control Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Dog No</td>
<td>1  2  3  6  7  4  9  10  11  12  13  14  20  21  22</td>
<td>8  16</td>
<td>5  2  18  1  2  25</td>
</tr>
<tr>
<td>No of CVL signs</td>
<td>2  2  1  3  1  1  2  2  2  3  1  1  1  3  2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The group depending of severity of CVL</td>
<td>Group (1)(Single CVL signs)</td>
<td>Group (2)(Tow CVLclinical signs)</td>
<td>Group (3)(&gt;3 signs)</td>
</tr>
<tr>
<td>1  3</td>
<td>20  4  3  14  7  1  2  10  22  11  9  6  12  21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Fe  M</td>
<td>Fe  M</td>
<td>Fe  M</td>
</tr>
<tr>
<td>The gender total no</td>
<td>2  4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Experimental dogs total no</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Ratio of severity</td>
<td>18%</td>
<td>27%</td>
<td>36%</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>
Ulceration: five dogs out of seventeen (29.4%) showed a typical CVL lesions two months post infection, plate (14).

Alopecia: two dogs out of seventeen (11.8%) had an alopecia, plate (15).

Weight loss: Eleven dogs out of seventeen (64.7%) showed weight loss associated with a low appetite, plate (16). In Figure (6), the average of clinical scores in the experimental dogs is shown.

![Major clinical signs of CVL](image)

RCS project, University of Khartoum, 2009

Figure (7): The percentages of major clinical signs of CVL were observed in this study.
4.2.1. Coat appearance

Plate (6): Hair loss of infected dogs.

Plate (7): Change of Furs’ colour of infected dogs.

4.2.2. Conjunctivitis manifestation

Plate (8): The conjunctivitis manifestation of infected dog.

4.2.3. Bone abnormality

Plate (9): Bone abnormality of infected dog.
4.2.4. Onychogroposis:

Plate (10): Normal black nails of dogs.

Plate (11): Change of nails’ colour of infected dogs.

Plate (12): Normal length of dog’s nails.

Plate (13-A) and Plate (13-B): Elongation of infected dogs' nails.

4.2.5. Ulceration:

Plate (14): The ulceration in different areas of the body of infected dogs.
4.2.6. Alopecia:

Plate (15): The alopecia of infected dogs.

4.2.7. The loss of body weight:

Plate (16): The loss of body weight of infected dogs.
4.3. Haematological findings

4.3.1. Blood haematology (smear)

Venous blood was smeared on forested slide and was investigated under Olympus microscope (high magnification (40x) the results were showed that (70.5%) of infected dogs had anaemic RBCs Normocytic – Hypochromic type, plate (17) shows the normal shape of Dog’s Red Blood Cells, plate (18) shows the anaemic one.

Plate (17): Shape of infected dog’s Red Blood Cells (0 time, normal shape).

Plate (18): Shape of infected dog’s Red Blood Cells three months post infection (anaemic RBCs).

4.3.2. Blood Haematology (Analysis)

All control dogs had normal level of blood cells count, (95 %) of infected dogs (16 out of seventeen) showed continuous decrease (monthly) of blood cells number, figures ( 7, 8, 9, 10, and 11) represent the results of blood haematology data throughout this study.
4.3.2.1. Red Blood Cells count

The results of RBCs count analysis were showed that there was no significant difference between the control and infected dogs through out the experiment periods, (figure 8).

![Red Blood Cells count chart](image)

**Figure (8):** Comparison of Red Blood Cells count between the infected dogs and control.

4.3.2.2. White Blood Cells count

White Blood Cells analysis results were showed a significant difference between a control and infected dogs in the count, (figure 9).

![White Blood Cells count chart](image)

**Figure (9):** Comparison of White Blood Cells count between the infected dogs and control.
4.3.2.3. Platlets analysis

Platlets analysis results were showed a significant difference between a control and infected dogs in the count, figure (10).

![Platelets analysis graph](image)

**Figure (10):** Comparison of platelets count between the infected dogs and control.

4.3.2.4. Package Cells Volume

The results of PCV value analysis were showed that, there was a significant difference between the control and infected dogs through out the experiment periods.

![Package Cells Volume graph](image)

**Figure (11):** Comparison of Package Cells Volume value between the infected dogs and control.
4.3.2.5. Haemoglobin

The results of Hemoglobin estimation were showed that there was a significant difference between the control and infected dogs throughout the experiment periods.

![Figure (12): Comparison of Hemoglobin estimation between the infected dogs and control.](image)

4.4. The Body weight

The results of the body weight data analysis were showed no significant difference between the control and infected dogs groups.

![Figure (13): Comparison of body weight loss between the infected dogs and control.](image)
4.5. Parasitological investigation (lymphnode smear)

Popliteal lymph nodes were enlarged in some dogs (13/17) (21%), three months post infection. Parasitological examinations of these dogs were carried out by popliteal lymph node tissue biopsy, and preparations stained with Giemsa stain, were examined by Olympus microscopy (40 x), amastigotes were seen in three slides out of seventeen (17.7 %), plate (19). All culture results were negative.

Plate (19): The amastigotes in lymphnode smear.

4.6. The PCR results

All samples obtained from control group showed negative results with all primer pairs through out the experiment. It is a variation of sensitivity between the four primers used in the study with infected group, table (5). RV1-RV2 kinetoplast primer pair showed (+ve) result with all infected dogs (symptomatic & asymptomatic). Table (6) shows the PCR results for RV1-RV2 primer pair compared to other primer pairs used in the study.
**Table (5):** The sensitivity of the four primers used in the study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>No of (+ve) Dogs</th>
<th>No of (-ve) Dogs</th>
<th>The ratio of sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV1-RV2</td>
<td>17</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>Pia1-Pia2</td>
<td>12</td>
<td>5</td>
<td>70.5%</td>
</tr>
<tr>
<td>AJS3-DB8</td>
<td>11</td>
<td>6</td>
<td>64.7%</td>
</tr>
<tr>
<td>Pia3-Pia4</td>
<td>7</td>
<td>10</td>
<td>41.1%</td>
</tr>
</tbody>
</table>

**Table (6):** RV1-RV2 kinetoplast primer pair in sensitivity compared to the other three primer pairs used in the study.

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>RV1-RV2</th>
<th>Pia1-Pia2</th>
<th>AJS3-DB8</th>
<th>Pia3-Pia4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>416</td>
<td>0</td>
<td>416</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>44</td>
<td>92</td>
<td>105</td>
<td>31</td>
</tr>
<tr>
<td>P value</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.000</td>
</tr>
</tbody>
</table>


4.6.1. Kinetoplast primer pair (RV1-RV2)

All infected dogs were showed (+ ve) results with specific primer pair RV1-RV2 after 16 months post infection (the band size 145bp) indicating the presence of *Leishmania donovani* parasites, figures (14).

![Image](image.png)

**Figure (14 .A) :** Shows the results with specific kinetoplast primer pair RV1-RV2:
Lane (1&4) -ve control, lane (2,3, and 5) +ve control read band with size 145 bp, lane (6-10) dogs’ samples (Apr /08), lanes 12, and 13 samples of dog Feb /08, lane 14, 15, and 16 samples of dog (March /08), Lane (11) Moleclar weightmarker (100bp).
B. Samples screening by RV1-Rv2 Kinetoplast primer pair

Figure (14.B): shows +ve results with all infected dogs (June/08). Lane (1) MW (100bp), lane (2) –ve control (primer diamér), lanes (3 to 12) sample of infected dogs, lane (13) +ve control, lanes (14, 15, 16) sample from control dogs.

4.6.2. Genomic Primer pair Pia1-Pia2:

All symptomatic dogs (88.2%) (15/17) were showed (+ve) results with Pia1-Pia2 primer pairs with band size 100bp, asymptomatic dogs and control were showed (–ve) results, figures (15) present the results.

Figure (15.A): Shows the results by Pia1-Pia2 genomic primer pair:

MW= Molecular weight marker (100 bp), lane (1,2) asymptomatic dogs’ samples, lanes (3-14) infected dogs’ sample, lane (15) +ve control, lane (16) –ve control.
A-Dogs’ blood sample screening by Pia1-Pia2 genomic primer pair:

Figure (15.B): presents samples screening by pia1-pia2 primer pair. Upper row: lane (1) -ve control, lane (2) +ve control band size (100bp), lanes (3 to 15) infected dogs’ sample. Lower row: lanes (1 to 6) infected dogs’ sample, lanes (7 to 15) controls’ sample.

4.6.3. Kinetoplast primer pair AJS3-DB8:

It was low sensitivity, it showed (–ve) results with asymptomatic dogs (Two out of seventeen) and four dogs from symptomatic ones, so the total of (-ve) results with AJS3-DB8 six dogs out of seventeen (35.3%), and showed (+ve) results with eleven dogs (64.7%), figure (16) shows the results.

Figure (16): lane (1) molecular weight marker (100 bp), lane (2) –ve control, lane (3) +ve control (800 bp) from VL patient, lanes (4 to 11) blood sample from infected dogs (March/08), lane (12) pool of samples obtained from the control dogs, lanes (13 to 16) samples from infected dogs (April/08).
4.6.4. Genomic Primer pair Pia3-Pia4:

**Figure (17.A):** lane (1) +ve control (120 bp), lane (15) –ve control, lanes (2 to 9) samples from infected dogs (March/2008), lane (10) sample from control group (pool), lanes (4,5) samples from asymptomatic dogs (8+16), lanes (11-14) samples from infected dogs (May\2008), lane (16) Molecular weight marker. All +ve results with primer diamer.

**Figure (17.B):** Shows the results by Pia1-Pia2 genomic primer pair: MW= Molecular weight marker (100 bp), lane (1, 2) +ve control, lanes (3&4) sample from asymptomatic dogs, lanes (5-14) samples from infected dogs, lane(15)-ve control.
4. 6.5 Comparison between the two genomic primer pair:

Figure (18) shows the comparison between two genomic primer pairs results (pia1-pia2 band size =100bp, and pia3-pia4 band size=120bp), and sensitivity of each one.

**Figure (18):** Shows the comparison between Pia1-Pia2 & pia3-Pia4, lane (1) Molecular weight marker (100bp), lanes (2 to 10) Dogs’ sample by pia3-pia4, lanes (11 to 16) Dogs’ sample by pia1-pia2.
CHAPTER FIVE

DISCUSSION

Within the context of WHO-TDR funded project (2005-2008), which aims at incriminating a reservoir host of visceral leishmaniasis in Eastern Sudan. The present study attempted an experimental infectivity of some indigenous dogs (Canis familiaris) with Leishmania donovani to know the likelihood of infection and sequentially the onset of the canine visceral leishmaniasis (CVL).

Currently, Leishmaniasis is increasing worldwide, principally due to increases in human migration and travel. This phenomenon increases the likelihood of leishmanial infection in low or nonendemic areas (Lainson and Rangel, 2005). The success of leishmaniasis transmission control programs depends on several preventive measures, such as surveillance of reservoirs, insect vectors and population.

In Brazil, one approach used to reduce the incidence of human VL is to cull infected dogs (Aparecida et al., 2007). The impact of such dog-culling programs on incidence of human and canine VL has been questionable and controversial (Reithinger et al., 2002b; Capelli et al., 2004).

The role of the dogs as a reservoir host of human visceral leishmaniasis has been recognized since Nobel Prizewinner, Charles Nicole’’s discover of the disease in dogs in Tunisia in 1908. (Killick-Kendrick.1999).
Studies have shown that the prevalence of infection is much higher than previously supposed, and that dogs with apparent infections are able to infect sandflies and play a part in the circulation of the parasite. (Killick-Kendrick. 1994).

In the Mediterranean region, dogs and wild canids are considered and proven to be the main reservoir of Human and Canine Visceral Leishmaniasis (Abranches, 1984, Peters & Killick-Kendrick. 1987).

The seroprevalence of canine leishmaniasis in areas of endemicity in the Mediterranean basin ranges between 10 and 30% (Solano-Gallego et al., 2001, Zaffaroni et al., 1999). However, most infected dogs do not present any clinical signs (Cabral et al., 1998; Sideris et al., 1999), and there is evidence that the infection prevalence rates are higher than those given by serological study when molecular tools were applied (Lachaud et al., 2000).

In Sudan and other places in East Africa, the zoonatic transmission cycle of *Leishmania donovani* is not well understood (Jamjoom et al., 2004). A number of investigations have been carried out in East Africa to find wild and/or domestic reservoir hosts of human VL. In Eastern Sudan, Bousfield in 1911 found a single dog with amastigotes of *Leishmania* in Gallabat area (Dereure et al., 2003). In 1924, Archibald and Susu (cited by Kirk, 1956) described one positive dog in Kassala. In 1956, R. Kirk found a single positive dog out of fifteen in Tapotha. Also, in Kapoeta south of Sudan, Zaki related an unconfirmed report of two infected dogs (Hoogstral & Heyneman, 1969). All dogs examined in Eastern Sudan by Jaques Dereure et al., 2003 were found to be
negative on serological examination grounds. The domestic dog may be an important reservoir host of *L. donovani* in Eastern Sudan in a recent study by Hassan *et al.*, 2009, who used serology & PCR technique.

The first mention of specifically identified *Leishmania donovani* in dogs was made by Mutinga *et al.*, 1980, who isolated two stocks from Kenya dogs, (Moreno *et al.*, 1986). In Iraq, another canine strain was isolated by Sukkar *et al.*, 1981, was identified as *Leishmania donovani* MON-3 (Moreno, *et al.*, 1986).

Within an epidemiological survey carried out in Eastern Sudan, identification of *Leishmania infantum* and *L. archibaldi* strains was reported in dogs (Dereure *et al.*, 2000). The same group also reported the presence of sympatric *L. donovani, L. infantum* and *Leishmania archibaldi* in a man and a dog (Dereure *et al.*, 2003).

Dogs are usually used as a model for the study of human VL, as well as for the development of new prophylactic and therapeutic programmes since canine VL is similar to the human disease.

All intravenously infected dogs were found to harbour parasite, indicating that the experimental infection with $4 \times 10^8$ stationary phase promastigotes was 100% successful in establishing a persistent infection. The infection rate is slightly higher than the average rate reported in literature for i.v. infection. Intravenous infection of dogs with amastigotes resulted in 87% average infection rate (n=30) (Campino *et al.*, 2000; Leandro *et al.*, 2001), and infection with promastigotes resulted in infection of 90% of dogs (n=58) (Abranches *et al.*, 1991; Campino *et al.*, 2000; Santos-Gomes *et al.*, 2002). The difference between the
average reported rate and the 100% infection here found is probably due to a combination of factors: 1) Parasite virulence characters. 2) The method of parasite detection: compared to direct examination of smears and PCR of blood sample, is a more sensitive methods; 3) The duration of the experiment: some of the dogs in the experiment were first found parasite positive at two months p.i. Together, the data indicate that an intravenous infection with stationary phase promastigotes has a very high (if not 100%) success rate and a dose of $4 \times 10^8$ per dog is sufficient.

Fifteen dogs out of seventeen (88.2%) developed clinical signs of Canine Leishmaniasis. The first signs appeared within the first two months post infection. Two dogs (11.8%) remained asymptomatic during the course of study, similar findings were reported by Jacqueline Poot, (2006). The overall loss in body weight was observed after 8 weeks post infection, similar observation has been reported in the human VL cases by many researchers reviewed in (Osman, 2000).

In this study skin and coat abnormalities are the most usual manifestations, and several dermatological entities have been observed, that similar as described by (Ferrer et al., 1988).

Also the gender ratio for the number of female to male dogs were almost the same (9 males: 8 females), female dogs showed more severe clinical manifestations. Infact the sample size was small to draw a conclusion on this observation, but future investigation should give close attention to wether females are more susceptible and more immune suppressed compared to males.
The hematological findings showed that 12 out of 17 dogs (70.5%) had anemia, and blood parameters (blood cells count, hemoglobin estimation, and package cells volume) showed significant value when compared with sample obtained from control group ($p > 0.05$), a similar result was found by Jacqueline Poot et al., (2006). In this study microscopic examination of blood smears did not show positive results (parasites were not observed). The result was expected as many research teams, showed low sensitivity for microscopy in peripheral blood (Zijlstra et al., 1992; Osman, 1997).

The detection of *Leishmania* DNA was possible by PCR (Belli et al., 1998; Rodgers et al., 1990). Several studies have shown the interest of PCR for the detection of CVL. (Berrahal et al., 1996; Roura et al., 1999; Zerpa et al., 2000). Suspect samples are analyzed by parasitological, haematological tests which were unable to distinguish *Leishmania* species. Consequently, sensitive and rapid diagnostic tests need to be considered. Therefore, PCR was chosen to detect *L. donovani* in biological samples using specific and sensitive primers because other methodologies, such as hybridization, biochemistry characterizations and RFLP, have shown high sensitivity and specificity (Schallig and Oskam, 2002; Volpini et al., 2003), but are timeconsuming, and costly methods.

The primary objective was, to detect the *Leishmania* in dogs by PCR assay using peripheral blood. Indeed, this type of sampling (Peripheral blood) has the advantage of being stress free and easily repeatable compared with bone marrow, skin, or lymph node sampling, the lysis and extraction methods had to be specifically optimized after many trials in order to reduce the inhibition rate and improve the specificity of the
reaction. The concentration of DNA was suitable (DNA quantitation was done), the volume of DNA that was used in study was sets modified several times, to optimize the PCR reaction, since the parasite concentration in peripheral blood was very low.

In this study four primers were used, with the aim for finding the most efficient for detecting the parasite in dogs. All methods detected the Leishmania parasite, but the sensitivity varied considerably according to the method used. Two methods targeting genomic DNA varied in sensitivity. kDNA minicircles are good candidates since they present very high copy number (10,000 per cell) and contain conserved and variable sequences so two methods were used. On the whole, the sensitivities of primers in this study were higher than (or equal to) those reported by other authors using the same primer pairs (Merdith et al., 1993; Quinnell, 2001. Zerpa et al., 2000).

The kinetoplast (AJS3-DB8) was less sensitivity, showing that PCR sensitivity does not necessarily correlate to the degree of replication of the DNA target. This has also been noted by Reithinger et al., (2000) with different kinetoplastic primer sets.

The other kinetoplastic primer RV1/RV2, proved extremely sensitive, interestingly and was used in this study because it has ability to amplify a sequence of the specific region for the Leishmania donovani complex. Moreover, the high sensitivity (at least one parasite in 5–10 ml of blood)
and specificity of these primer in blood samples from dogs with visceral leishmaniasis in the Mediterranean rendered them strong candidates for the present study purpose (Le Fichoux et al., 1999; Lachaud et al., 2002a). Here, RV1/RV2 primers were also specific when reference strains were assayed. As regards specificity and practicability, method Db8-Ajs3 was considered inadequate for *Leishmania* detection in dogs.

The genomic primer pair Pia3-Pia4 was found impractical but was kept for further evaluation. The remaining genomic DNA-based method Pia1-Pia2 did not prove sensitive enough with asymptomatic dogs.

The PCR results showed high rate of sensitivity that similar to the PCR results reported by Jaqueline Poot, 2006, who found that 100% of experimentally infected dogs were +ve by kinetoplastic primer pair. The PCR results found in this study does not match with the recent finding by study carried out in Eastern Sudan (natural infection) by Hassan et al., (2009). Who found that two dogs out of 87 (2.3%) were PCR +ve result by specific primer pairs. These differences may be due to the following aspects: the experimental infection (the present study), was carried out with high inoculums of cultured promastigotes from the stationary phase, which refers the total number of promastigotes forms and not only virulent metacyclic-like promastigotes and inoculums used here does not include saliva of the insect vector (natural infection).

The work described here demonstrates that the inoculum of cultured promastigotes by intraveinously (IV) in canine model (*Canis familiaris*) is successful in transformation of promastigotes into amastigotes which are phagocyted by macrophage cells, the incubation period was 16
months, this similar with Killick-Kendrik et al., 1994, but it was longer compared to intradermal (ID) inoculation (6-14 weeks, Vidor, et al., 1991).

**Conclusion:**

In conclusion, intravenous infection with promastigotes appears to be a useful model for the establishment of reliable and rapid infections in dogs. Such trials can be valuable in Leishmaniasis control programmes, disease pathology, and vaccine efficacy. For evaluation of the effect of experimental infections, the direct detection of parasites (microscopy and/or PCR) is the most sensitive and reliable parameter.

**Recommendations:**

- Canine Leishmaniasis experimental infection is important and can be used in future programmes for vaccine studies evolution and monitoring of chemotherapy under use and for evolution of new prescriptions.
- In future studies, verification of the continuity of the transmission should be tested by feeding colony-reared sandflies on these experimentally infected dogs.
- Since infection can progress to the CVL disease, therefore the parameters and features described in this study can be used as standard markers for canine reservoir host survey in the endemic areas of the disease.
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## Appendix A

Table (1): The haematological parameters analysis:

<table>
<thead>
<tr>
<th>Variable</th>
<th>RBCs (Mean±SD)</th>
<th>WBCs (Mean±SD)</th>
<th>Platelets (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3818857±182148 0 6047143±141823 5 6932428±1415926</td>
<td>10700±701 8.31 15382.85±4838 .73 25645.61±25597. 64</td>
<td>466142.4±500 07.37 430463.9±4527 89.91 279171.2±38221 4.53</td>
</tr>
<tr>
<td>Infected</td>
<td>4833529±966714. 5 6468235±103300 9 7185882±599 6724</td>
<td>7776.47±16 39.18 17062.5±13562 .99 14753.53±5012.6 5</td>
<td>141823.4±22.8 88.19 314975.2±4020 47.45 228670.5±31199 5.73</td>
</tr>
<tr>
<td>p-value</td>
<td>0.3</td>
<td>0.005</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table (1): The haematological parameters analysis (cont)

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCV (Mean±SD)</th>
<th>Hb (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>2006</td>
<td>2007</td>
</tr>
<tr>
<td>Control</td>
<td>586.6±514.37</td>
<td>302.03±430.6</td>
</tr>
<tr>
<td>Infected</td>
<td>205.65±378.59</td>
<td>258.88±400.39</td>
</tr>
<tr>
<td>p-value</td>
<td>0.10</td>
<td>0.123</td>
</tr>
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</table>
Table (2): The body weight analysis throughout the periods of study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Weight (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2006</td>
</tr>
<tr>
<td>Year</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.8±3.63</td>
</tr>
<tr>
<td>Infected</td>
<td>8.66±2.47</td>
</tr>
<tr>
<td>p-value</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Appendix B

Clinical signs of Canine Visceral Leishmaniasis.

<table>
<thead>
<tr>
<th>No</th>
<th>Major clinical signs seen in CVL</th>
<th>No of Dogs with CVL signs</th>
<th>Percentage of Dogs with CVL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lymphadenomegaly</td>
<td>13</td>
<td>76.5%</td>
</tr>
<tr>
<td>2</td>
<td>Splenomegaly</td>
<td>7</td>
<td>41.2%</td>
</tr>
<tr>
<td>3</td>
<td>Fever</td>
<td>4</td>
<td>23.5%</td>
</tr>
<tr>
<td>4</td>
<td>Ulceration</td>
<td>5</td>
<td>29.4%</td>
</tr>
<tr>
<td>5</td>
<td>Onychogryposis/Abnormal nails(extention+color)</td>
<td>(7+3) 10</td>
<td>58.8%</td>
</tr>
<tr>
<td>6</td>
<td>Conjunctivitis</td>
<td>1</td>
<td>5.9%</td>
</tr>
<tr>
<td>7</td>
<td>Weigh loss</td>
<td>11</td>
<td>64.7%</td>
</tr>
<tr>
<td>8</td>
<td>Hair loss</td>
<td>2</td>
<td>11.8%</td>
</tr>
<tr>
<td>9</td>
<td>Fur change color</td>
<td>3</td>
<td>17.6%</td>
</tr>
<tr>
<td>10</td>
<td>Unnormal locomotion (bones problem)</td>
<td>2</td>
<td>11.8%</td>
</tr>
<tr>
<td>11</td>
<td>Alopecia</td>
<td>2</td>
<td>11.8%</td>
</tr>
</tbody>
</table>