STUDIES ON THE TRANSMISSION DYNAMICS OF
LEISHMANIA DONOVANI IN GADARIF STATE
EASTERN SUDAN

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ABSTRACT

This study was carried out in eastern Sudan by introducing dogs from non endemic area to VL endemic area to elucidate the natural *L. donovani* infection in dogs (*Canis familiaris*, Linnaeus, 1758) and then to determine their role in transmission of visceral leishmaniasis as reservoir hosts.

This study comprised 3 groups of dogs, the first one was the natural *L. donovani*, infection test group, consisted of 35 dogs selected with the age of less than 6 months, from non endemic area of V.L. (Khartoum) transferred to the VL. endemic area (17 dogs in Tabarak Alla and 18 in Barber el fugara). The second group was healthy controls consisted of five dogs kept at Khartoum State, in the Police Dogs Directorate. The third group consisted of 25 local dogs sampled randomly during a survey done in December 2008 – Feb. 2009. All dogs from group 1&2 were checked free from *Leishmania donovani*. They were vaccinated with routine vaccines (Distemper, canine adenovirus type 2, canine parvovirus and rabies).

The field study was continued for more than 2 years (32 months) (June 2006 – February 2009). Trips for sampling and data collection were regulated every (1-2) months. Dogs were tested for the development of symptoms and signs of CVL on monthly basis. Venous blood and lymph node, bone marrow aspirates were collected for the detection of infection using the PCR, DAT, microscopy and culture. The results showed that, the transferred dogs group (1) had developed typical clinical signs of CVL like, lymphadenomegaly, long nails, weight loss, & different skin lesions. Parasitological examination showed the presence of the distinctive promastgote forms in cultures of BM& LN in
(18.2%) of transferred dogs, which also showed (55.6%) and (100%) of PCR & DAT positive results respectively. In the other hand, the local dogs, although they showed no clinical symptoms, they were (84%) & (96%) positive by PCR& DAT respectively. In addition to these results, questionnaires designed to know the relation of keeping domestic animals including dogs & the VL infections in human. Showed a positive contribution of them to VL risk factors.

It was concluded that dogs can acquire natural infection with *Leishmania donovani*, and thus may be considered as a potential reservoir of leishmaniasis that can play a significant role in VL transmission in that endemic area.
Canine Visceral Leishmaniasis (Canis familiaris) is a disease transmitted by sandflies, which causes symptoms in dogs. The study's objective was to investigate the incidence of this disease in the North of the Sudan. A group of 35 households was divided into three categories: the first included 32 households that were monitored for eight months, the second included five households that were monitored for two years, and the third included 25 households that were monitored for one year. The study was conducted from 2006 to 2009. The incidence of leishmaniasis in dogs was investigated in the North of the Sudan. The study found that the disease is prevalent in the region and that it affects a significant number of dogs. The study also highlighted the importance of monitoring the disease to prevent its spread.
مثال ل Vuexaria 19.2% و التغييرات الوزنية فعان الكشف أوضحت النمط التفاعل البوتاليك بإنجاب النتائج بسبب البروتينات 8 و 96% في الفحص ببوتاليك بعينة أظهرت النتائج تحسين النمو في الشيخوخة متكافئة بين وثيقية العلاقة ووجود بالمرض إصابات و بالمنازل الأخرى للحيوانات بالة والنظام إلى أن تكون قد حمل دور الانتشار في الانتقال والخشونة وبداية 100% إزاء النمو ل الدكتور: يوشوك غودرال وت.’
Dedication

I dedicate this work to my parents,
My husband, daughters & son
Moreover, all my friends and colleagues.
ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Omran F. Osman for valuable supervision, patience, help and encouragement.

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It was concluded that dogs can acquire natural infection with *Leishmania donovani*, and thus may be considered as a potential reservoir of leishmaniasis that can play a significant role in VL transmission in that endemic area.
المستخلص

أجريت هذه الدراسة بشرق السودان بولاية القضارف لمعرفة عما إذا كانت الليشمانيا الحشوية يمكن أن تصيب الكلاب وتظهر عليها أعراض مرض ليشمانيا الكلب الحشوية (Canine Visceral Leishmaniasis)، كذلك هدفت التجربة لدراسة دور الكلاب كعائل وسيط (خازن للطفيل) في انتشار مرض الليشمانيا الحشوية.

اشتملت هذه الدراسة على ثلاث مجموعات من الكلاب:

المجموعة الأولى تشمل 35 من الكلاب لا تزيد أعمارها عن ستة أشهر جمعت من ولاية الخرطوم المعروفة بوجود داء الليشمانيا الحشوية فيها ونقلت إلى المنطقة المطلقة بداء الليشمانيا الحشوية ووزعت علي قريتي باردك (17) وبربر القرواء (18) لدراسة الإصابة الطبيعية بالطفيل المسبب للشيرمانيا الحشوية.


كل الكلاب من المجموعة الأولى والثانية فحصت للتأكد من خلوها من الطفلك المسبب لداء الليشمانيا الحشوية، كما حصنت من الأمراض الفيروسية والوبائية مثل (السحار، السل الكلاب، التهاب الكبد و أمراض الجهاز التنفسي).

استمرت الدراسة الحقلية لأكثر من عامين (حوالي 32 شهرا) في الفترة من يونيو 2006 إلى فبراير 2009.
استمرت معاينة الكلاب شهرياً للكشف عن ظهور أعراض مرض ليشمانيا الكلاب الحشوية 
أخذت عينات دم من الوريد وسحبت سائل ال عق الليمفاوية ونخاع العظام بغرض الكشف 
عن الطفيلي باستخدام تقنية التفاعل التسلسي بإنزيم بوليميريز، التشخيص ألمجريي 
والترزيع الطفيلي واختبار التلاذن المباشر. أظهرت الكلاب التي تم نقلها إلى المنطقة التي 
يتواجد فيها داء الليشمانيا الحشوية أعراض مرضية مطابقة للأعراض الإكلينيكية لمرض 
ليشمانيا الكلاب الحشوية، مثل: تضخم العقد الليمفاوية، استطالة في المخلب، فقدان 
الوزن والتغيرات المرضية الجلدية المختلفة. كما أوضح الكشف عن الطفل نتائج إيجابية 
في التزريع من سائل الغدد الليمفاوية ونخاع العظام بنسبة (18.2%)(55.6%) بتقنية 
التفاعل التسلسي بإنزيم بوليميريز (100%) باختبار التلاذن المباشر.

في الجانب الآخر أظهرت الكلاب المحلية المتواجدة بالمنطقة التي يتوطن فيها داء 
الليشمانيا الحشوية إيجابية بنسبة 84% و 69% بفحص التفاعل التسلسي بإنزيم 
بوليميريز واختبار التلاذن المباشر.

أثبتت نتائج الاستبيانات التي جمعت من المسح الذي أجري لمعرفة علاقة تربية الكلاب 
والحيوانات الأليفة الأخرى بالمنازل والإصابة بالمرض وجود علاقة وثيقة بين تربية 
الكلاب والحيوانات الأليفة بالمنزل والإصابة بداء الليشمانيا الحشوية.

كل هذه النتائج مجتمعة تجعلنا نستنتج إجابة أن يكون الكلب عامل وسيط خارج للطفل له 
دور في انتشار و انتقالية طفل الليشمانيا الحشوية بتلك المناطق المستوطنة بالمرض.
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Leishmaniasis:
Leishmaniasis is one of the most diverse and complex of all vector borne diseases because it involves several overlapping species and sand fly vectors, the disease has a complex ecology and epidemiology (Sharma & Singh, 2008).
Leishmaniasis has been considered tropical afflictions that together constitute one of the six entities on the World Health Organization/Tropical Disease Research (WHO/TDR) list of most important diseases. The disease is endemic in 88 countries in five continents with a total of 350 million people at risk and annually 12 million cases are reported.
Of the 88 endemic countries, 22 are in the New World and 66 in the Old World with an estimated incidence of about 1.5 million cases of cutaneous leishmaniasis (CL) and 500,000 cases of VL per year (Singh, 2006). More than 90% of the CL cases occur in Iran, Afghanistan, Syria, Saudi Arabia, Brazil, and Peru. Of the 500,000 new cases of VL, more than 90% are reported from India, Nepal, Bangladesh, Sudan and north-east Brazil.

1.2. The genus Leishmania:
The causative agent Leishmania belongs to the kingdom: Protista, phylum: Euglenozoa and family: Trypanosomatidae (Sharma &
Singh, 2008) *Leishmania* (Kinetoplastida) are protozoan parasites of great medical and veterinary significance (Dantas-Torres, 2006b), which are transmitted to a susceptible host by Phlebotomine sand flies (Diptera : psychodidae) of the genera *Phlebotomus* and *Lutzomia* in the Old and New World, respectively. The genus *Leishmania* is divided into two subgenera, *Leishmania* and *Viannia*, based on the pattern of development of the parasites in the sand fly gut (Lainson and Shaw, 1987). Of some 30 species of *Leishmania* known at present, ~ 20 are pathogenic for humans. About 30 species of Phlebotomine sand flies are proven vectors (Ashford, 2000; Desjeux, 2004).

### 1.3. Life cycle of *Leishmania*:

The life cycle of *Leishmania* is simple and it involves two stages without sexual stage. (Figure 1). In insect vector, it takes a promastigote form, which is characterized by, elongated, motile and an extracellular stage, while in vertebrates the parasite is found in amastigote form. The amastigotes are ovoid non-motile and intracellular stage. The insect vector injects promastigotes into the host’s skin and soon after the parasite is taken up by skin macrophages, where the promastigotes transform into amastigote form within 12-24h of inoculation. After transformation, the amastigotes multiply within the macrophage and ultimately the macrophage bursts releasing the amastigotes to infect other macrophages. This stage is chronic in nature and may continue for
months to years and even for lifetime without noticeable signs and symptoms, depending upon the host susceptibility and its immune status. The infected macrophages may remain localized to the skin, as in case of CL leading to ulcer formation, or may disseminate to other organs, as in VL or to mucosa as in mucocutaneous leishmaniasis (MCL).

The sand fly of 2-3 mm penetrates the host skin with its sharp cutting mouthparts from where small pools of blood oozes out, the sand fly feeds on this oozing out blood pool. It is postulated that in cases of CL, the infected macrophages also ooze out with the pool of blood and are taken up by the sand fly. In cases of VL, where the parasite is concentrated in the spleen, liver and bone marrow, some infected macrophages are released in the blood circulation and it is a chance that the same macrophage is taken up by the sand fly.

As the sand fly takes up the amastigotes, the transformation of amastigotes starts within hours of ingestion and completely transformed into motile promastigote within 24-48 hrs. and keep on dividing by binary division. The mature metacyclic promastigotes accumulate in the mid-gut and fore-gut.

The sand fly transmits the infection during another blood meal on the same or another host species. (Sharma & Singh, 2008).
Figure (1): The life cycle of *Leishmania spp.* in mammalian host and vector.

From Wikipedia, the free encyclopedia (www.wikipedia.com).
1.4. Clinical manifestations:
The manifestations of Leishmaniasis depend on complex interactions between the virulence characteristics of the infecting *Leishmania* species and the immune responses of its human host. The result is a spectrum of disease ranging from localized skin lesions to diffuse involvement of the reticuloendothelial system. Human leishmaniasis presents in four different forms with a broad range of clinical manifestations. (Sharma & Singh, 2008).
Infection with *Leishmania* protozoa can result in cutaneous, mucocutaneous or visceral leishmaniasis, depending on the parasite, host, and environmental factors, (Bates, 2006).

1.4.1. Visceral leishmaniasis (VL):
Known also as Kala–azar (KA) is the most severe form of disease, which if untreated, has a mortality rate of almost 100%. It is caused by the species of *Leishmania Donovan* complex that consist mainly of *L.(d) infantum*, *L.(d) donovani* and *L.(d) chagasi*, (Sharma & Singh, 2008).
The visceral leishmaniasis, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anaemia, pancytopenia, and hypergammaglobuliaemia and is complicated by serious infections (Sundar& Rai, 2002).
Although confirmed cases of VL have been reported from 66 countries, 90% of the world’s VL burden occurs in the Indian

*Leishmania infantum* is responsible for VL in children in the Mediterranean basin. However, due to increasing prevalence of human immunodeficiency virus (HIV) in this region, HIV-VL co-infection in the adult population is being reported frequently. *Leishmania* –HIV co-infection is regarded as an emerging disease especially in southern Europe, where 25-70% of adults with VL have AIDS as well, leishmaniasis behaves as an opportunistic infection (Sundar & Rai, 2002).

1.4.2. Post kala-azar dermal leishmaniasis (PKDL):
Also known as "Post-kala azar dermatosis" (Rapini, et al., 2007). A cutaneous condition is characterized by a macular, depigmented eruption found mainly on the face, arms, and upper part of the trunk (James, et al., 2006).

Post-kala-azar dermal leishmaniasis (PKDL) appears as a dermatotropic form of the infection of this parasite as a sequel to kala-azar in 50% of the cases in Sudan and 10 to 20% of the cases in India (Ramesh, et al., 1995; Zijlstra, et al., 2000).

In Sudan and other East African countries, patients develop PKDL during or within 6 months after treatment for VL (El Hassan, et al., 1992; Muigai, et al., 1991; Zijlstra, et al., 2000). In Sudan, they can be demonstrated in up to 60% of treated cases. They can be manifested as hypo-pigmented macules, papules, nodules, or facial
erythema. Though any organism causing Kala-azar can lead to PKDL, it is commonly associated with *L. donovani*, which gives different disease patterns in India and Sudan (Zijlstra, & El Hassan, 2001). In the Indian variant, nodules enlarge with time and form plaques but rarely ulcerate, but nodules from the African variety often ulcerate as they progress. Nerve involvement is common in African variety but rare in Indian subcontinent. (Salotra, and Singh, 2006).

### 1.4.3: Cutaneous leishmaniasis:


It is the most common form of leishmaniasis. It is a skin infection caused by a single-celled parasite that is transmitted by sand fly bites. Cutaneous leishmaniasis is endemic to many parts of the world. Around twenty different species of *Leishmania* parasites are capable of infecting humans.

It is caused by *L. major, L. tropica, L. mexicana, L. amazonensis, L. guyanensis, L. panamensis*. The cutaneous leishmaniasis produces large numbers of skin ulcers, as many as 200 in some cases, on the exposed parts of the body. In general, half of these lesions caused by *L. major* or *L. Mexicana* heal in 3 months, those caused by *L. tropica* take about 10 months and those due to *L. braziliensis* persists much longer (Hepburn, 2003) 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).

In Sudan cutaneous leishmaniasis spread throughout the Northern Sudan along the Nile to Dongula especially in Khartoum. All age groups were affected with more than 10,000 cases reported in hospitals in Khartoum State (El-Safi, et al., 1991).

Recently, *Leishmania donovani* were identified as a cause for CL in the Sudan (El-Amin, et al., 2008), and Sir Lanka (Yamuna, et al., 2007).

1.4.4. Mucocutaneous leishmaniasis (MCL):  
The MCL (uta or espundia), produces lesions, which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities (Zijlstra, and El-Hassan 2001). The causative species of MCL are *L. (viannia) braziliensis* and *L. (viannia) guyanensis*, (Desjeux, 1996; WHO, 1996). Mucosal lesions have been reported in the Old World due to *L. donovani, L. major* and *L. infantum* in immuno-suppressed patients (Desjeux, 1996).

1.4.5. Diffuse cutaneous leishmaniasis (DCL):  
It is an anergic variant of localized CL in which lesions are disseminated, resembling lepromatous leprosy. The disease is caused by *L. (mexicana) amazonensis* and *L. aethiopica*, (Desjeux, 1996; WHO, 1996). It is less common, chronic in evolution and especially difficult to treat.
1.5. Inhabitancy 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 travellers, 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.

1.6. 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 are varieties 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 sand fly breeding and resting sites. (b) Type of housing. (c) Occupation. (d) Extent of exposure to sand fly 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 sand fly populations.
- Anthropophilia or zoophilia of sand flies and degree of it. Rarely, leishmaniasis is spread from a pregnant woman to her baby. Blood transfusions or contaminated needles also can spread leishmaniasis. Leishmaniasis is endemic to more than 80 countries and is an important public health concern with a global incidence of 1.5 – 2 million cases each year (World Health Organization, 2002).

1.7. The Geographic Distribution:

Leishmaniasis is a typical example of zoonosis found on all the continents except Australia and Antarctica (Anonymous, 1983.). The geographic distribution depends on the presence of sand fly vectors and animal reservoirs. Leishmaniasis has been considered tropical afflictions that together constitute one of the six entities on the World Health Organization/Tropical Disease Research (WHO/TDR) list of most important diseases. The disease is endemic in 88 countries on five continents with a total of 350 million people at risk and annually 12 million cases are reported. Of the 88 endemic countries, 22 are in the New World and 66 in the Old World with an estimated incidence of
~1.5 million cases of cutaneous leishmaniasis (CL) and 500,000 cases of VL per year. More than 90% of the cases of cutaneous leishmaniasis (CL) occur in Afghanistan, Algeria, Brazil, Iran, Pakistan, Peru, Saudi Arabia, and Syria; 90% of visceral leishmaniasis (VL) occurs in Bangladesh, Brazil, India, Nepal, and Sudan (Desjeux, 2004).

Despite this widespread geographic distribution, human leishmaniasis is often focal within an endemic area, leading to ‘hotspots’ of disease transmission. (Sharma & Singh, 2008).

The geographic distribution of leishmaniasis is cosmopolitan, figure (2).
1.8. The reservoir host:

With some exceptions, the leishmaniases are zoonosis and the human infection is incidental. Natural vertebrate hosts of *Leishmania* parasites are mammals of the orders: Edentata (e.g., armadillos, sloths), Carnivora (e.g., dogs, cats), Hyracoidea (e.g., hyraxes), Rodentia (e.g., rats, gerbils), Primates (e.g., humans, monkeys), Marsupialia (e.g., opossums), and Perissodactyla (e.g., horses) (Ashford, 1996; Saliba and Oumeish, 1999; Gramiccia and Gradoni, 2005). The primary reservoir hosts of *Leishmania* are sylvatic mammals, such as forest rodents and wild canids. With the increasing process of domiciliation of the zoonotic cycle of transmission of leishmaniases, synanthropic and domestic animals have assumed an important role as reservoirs of infection. Dogs have been found naturally infected by different species of *Leishmania*, such as *Leishmania (Leishmania) major* (Elbihari *et al*., 1987), although their role as reservoirs of some of these parasites is probably negligible; that is, they are more likely to be victims rather than reservoirs. In a recently published paper, Gomes *et al*., (2007) emphasize the utility of PCR in discriminating the species of *Leishmania* infecting dogs, particularly in areas where both visceral and cutaneous leishmaniases are endemic. Indeed, PCR based methods have been proven to be powerful tools for the detection – in different clinical specimens – and discrimination of *Leishmania* species infecting a wide range of vertebrate (Branda˜o-Filho *et al*., 2003; Oliveira *et al*., 2005; Silva *et al*., 2005; Andrade *et al*., 2006) and invertebrate hosts (e.g.,
Phlebotomine sand flies, ticks) (Coutinho et al., 2005; Parvizi et al., 2005).

Bhattarai et al., (2010) found *Leishmania* DNA in domestic animals (cows, buffalo and goats) from Dharan -17, in Nepal, mostly in goats (16%), cows (5%), and buffaloes (4%).

In East Africa, including Sudan, the transmission of *L. donovani*, is thought to be anthroponotic (Desjeux, 2001), especially during epidemic situations although zoonotic foci were encountered in these epidemics (Ashford, 1996). Apparently, the zoonotic transmission of VL in the region was initially observed following outbreaks of VL among people that camped in un-inhabited areas of eastern and southern Sudan (Kirk, 1956); Elnaiem et al., (2001) also observed high infection rates of *L. donovani* in uninhabited woodland areas in Dinder National Park (DNP) and provided evidence that in this habitat the Egyptian mongoose (*Herpestes ichneumon*) may be a primary reservoir host of the parasite. In a village habitat, infection in dogs was reported from the Atbara River area in eastern Sudan (Dereure et al., 2000; Dereure et al., 2003; Mukhtar et al., 2000). Dereure, et al., (2000) were the first to report firm evidence that the domestic dog may be an important reservoir of *Leishmania donovani* in eastern Sudan and other parts of east Africa.

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 techniques.
1.9. 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 (3). 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). In addition, 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. According to the reports of the Ministry of Health, Gedaref State, Sudan (2010), VL cases in endemic areas increased from 2751 in 2007 to 4000 cases in 2010.
Figure (3): Map of visceral leishmaniasis endemic zones of Sudan.
1.10. Diagnosis of leishmaniasis:

The diagnosis of visceral leishmaniasis is complex because its clinical features are shared by many other commonly occurring diseases for eg: malaria, typhoid, and tuberculosis (i.e. long-term unexplained fever, cachexia and hepatosplenomegaly etc.). Many of these diseases may be present with VL (co-infection cases) and sequestration of the parasite in the spleen, bone marrow, or lymph nodes may add to further complication.

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 lymph node, 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.

1.10.1 Parasitological methods:

1.10.1.1. Microscopy:

The routine diagnosis of leishmaniasis relies on the microscopic detection of *Leishmania* amastigote in Geimsa stained aspirate material 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 is no possibility to distinguish 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 1 study dating from the beginning of this century when 86% of the blood samples were found in the venous blood of seven of 20 confirmed VL patients.

1.10.1.2. 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 scrapings 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).

**1.10.2. Serological diagnosis (Immunological):**

Serological methods are highly sensitive and being non-invasive, they are comparatively more suited for diagnosing VL in endemic regions. These methods are either based on detection of antibodies (produced against parasite by polyclonal activation of B cells) or antigens. Currently, the most used methods for diagnosis of VL are direct agglutination test (DAT) and enzyme linked immunosorbent assay (ELISA) and immunofluorescent antibody test (IFAT).
1.10.2.1. Direct agglutination test (DAT):

Was introduced about two decades ago rapidly followed by its improved version for field use (Badaro et al., 1983; Zijlstra et al., 1992).

DAT has been found to be 91-100 per cent sensitive and 72-100 per cent specific in various studies elsewhere in the world (Zijlstra, et al., 2001; Vinayak, et al., 1994).

The test uses stained promastigotes either as a suspension or in a freeze-dried form. Problems such as the need of a cold chain for storage of antigen are avoided by using the freeze-dried antigen, which makes DAT very suitable for use under field conditions (Silva et al, 2005).

1.10.2.2. ELISA:

Enzyme-linked Immunosorbent Assay (ELISA) has been used as a potential sero diagnostic tool for almost all infectious diseases, including leishmaniasis. The technique is highly sensitive, but its specificity depends upon the antigen used. Several antigens have been tried like crude soluble antigen (CSA), fucose-mannose legend that is a 36-kDa glycoprotein present throughout the life cycle of Leishmania (amastigote and promastigote stages), and a recombinant antigen, rK39. (Sundar, and Rai, 2002).
1.10.3. Molecular diagnosis of leishmaniasis:

Polymerase chain reaction (PCR):

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 meres) which specifically bind to the DNA flanking the region of interest. The target sequences amplified using a heat-stable DNA polymerase isolated from thermos aquaticus (Saiki et al., 1988). First the double stranded (ds) target DN 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 gel electrophoresis and Southern 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 strict 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.

1.11. 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 cannot 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 lymph adenomegaly, 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 one 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).

**1.11.1. Geographical distribution:**

The geographical distribution of different *Leishmania* species is directly dependent on the local presence of the respective specific sand fly vectors. *L. 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; sero prevalence 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 based on genetic data (Mauricio *et al.*, 1999). In the new world, the parasite is transmitted by a different vector species: sand flies 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. Sero prevalence in dogs ranges from 24% to 67% in high endemic clusters (Miles *et al.*, 1999; WHO, 1990).

**1.11.2. Pre-patent period:**

Of the low numbers (1 to 100) of metacyclic promastigotes that are delivered with the bite of a sand fly, 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 pre-patent period
complicates epidemiological and intervention studies in the field as well as experimental infection models in the laboratory.

1.11.3. 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, & Ferrer, 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). 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.

1.11.4. 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 cruzi*, another protozoan that sometimes infects dogs in North America (Zandra *et al.*, 2000).

1.11.4.1. 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 erythematosus, skin diseases like demodicosis or dermatophytosis, endocrine disorders and malignancies like myelo-and lymphoproliferative diseases can also cause similar clinical manifestations.

1.11.4.2. 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 based either on identification of the parasite or on serology. The detection of parasites is the ‘gold standard’ for diagnosis; its sensitivity however is not high.

1.11.4.3. 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 amastigote in tissue samples is often very low, diagnosis can be difficult. The sensitivity of histopathology can be increased by applying immunohistochemistry 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).

1.11.4.4. 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).

1.11.4.5. 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 Immuno Sorbent Assay (ELISA). Evaluation revealed relatively high sensitivity (99.5-100%) and specificity (95-100%) without great differences between the different serological assays tested (El-Harith et al., 1989;
1.12. Justifications and objectives:

1.12.1. Justifications:

Gadarif State is one of the most endemic areas of visceral leishmaniasis in the Sudan and the disease which was localized in small belt is spreading dramatically over the past few years, involving Gadarif town itself and the neighbouring states. Visceral leishmaniasis is now number one public health problem in Gadarif State for the following reasons:

1- The incidence of the disease is on increase and is attaining epidemic levels (As in Tabarak Alla and Barber-elfugara areas). Previous reports indicated that at least 1,000 cases of V.L occur each year in Gadarif State (Osman et.al, 1997,) and according to the current reports of the Ministry of Health, Gedaref State, Sudan (2010), VL cases in endemic areas increased from 2751 in 2007 to 4000 cases.

2- The disease is spreading to areas in which V.L was unknown previously.

3- Difficulty in effective control measures against V.L because of the high cost of prevention and treatment and the epidemiology of leishmaniasis is insufficient. Thus attempt to control the disease would be almost impossible without any prior planning.

4- The disease affects the productive age groups.
1.12.2. Objectives:

1. To study the natural *Leishmania donovani* infection in dogs in the endemic area.
2. To compare the clinical features observed on the introduced dogs to those of the locally reared dogs within the endemic area.
3. To know the role of the dog in visceral leishmaniasis transmission.
4. To determine whether Visceral Leishmaniasis (VL) in this area is zoonotic or anthroponotic.
5. To study the role of rearing domestic animals (mainly dogs) in VL infection.
CHAPTER TWO
MATERIALS AND METHODS

2.1. Study area:
The fieldwork of this study was conducted in Gadarif State – Eastern Sudan, an area recognized as endemic for V.L. It involved two villages, Tabarak Alla and Barber elfugara, which are located close to Atbra River, Galabat locality (now Guraisha locality).
Tabarak Alla is about 97km southeast from Gadarif. Barber elfugara adjacent to Tabark Alla (see figure 5).
Tabarak Alla is inhabited by more than 4000 people of many tribes, mainly, Beni Amir, Masaleet, Zagawa, Bargue, Barnue, Birgid, Rashid, &etc.. The main inhabitants of Barber elfugara are from Tama tribe beside other tribes. Most of them are farmers and labourers in the agricultural schemes, growing sorghum, sesame, dukhun and groundnuts and breeding animals like cattle, sheep, goats and camels.
The landscape is generally flat and covered by cracked cotton clay soil and it is interrupted by some streams (khors) and meadows (mayaas).
The climate is tropical continental with an annual rainfall of about 800mm. The year may be divided into a hot, dry summer (March – May), a warm, wet autumn (June – October) and a moderately warm winter (November – February). The average temperature of the area is 18-45°C (Gadarif meteorological station, 2001).
The study area is characterized by reduced vegetation which is dominated by *Balanites aegyptiaca* (Higleeg), *Zyziphus spinachristie* (Sidir) and scattered *Acacia* trees within the villages (Elnaiem, et al., 2003).

The villages are surrounded by cultivated fields of Dura, Dukhun, Sesame and Groundnut.

The area is characterized by different animals like cattle, sheep, goats, donkeys, camels and dogs beside many rodents, squirrels and wild cats can be seen.

In Tabarak alla, the hospital was established by Zakat – Diwan for kala–azar diagnosis and treatment under the responsibility of Gedaref State Ministry of health, which also had established another health centre in Barber – elfugara.

The patients utilize the services of the two hospitals from about 23 villages around them like, Al assira, Mushraa El fursan, Khour zaraf, Jabal gana, Saraf aradeiba and other endemic areas.

People in these villages live in huts constructed of wood, bamboo and grass.
Figure (4): Satellite map showing the study area– eastern Sudan.
Figure (5): Study Area
Plate (one-a): vegetation in the study area

Plate (1-b): Cracked cotton clay soil.

Plate (1-c): Study area.

Plates (1): Study area. (Note huts& type of vegetation).
Plate (2): keeping domestic animals inside houses.

Plate (3-a): Tabark Alla hospital.  
Plate (3-b): Barber elfugara hospital.
Plate (3-c): Kala-azar patients in Tabarak Allah hospital.

Plate (3-d): a patient with PKDL.

Plate (four-a): test group dogs transferred from Khartoum.

Plate (4-b): one of the distributed dogs in Barber elfugara.
2.2. Study Design:

This study comprised three groups of dogs; the first one was the natural *L. donovani*, infection test group, consisted of 35 dogs selected with the age of less than 6 months, from non-endemic area of V.L. (Khartoum) transferred to the endemic area (17 dogs in Tabarak Alla and 18 in Barber elfugara).

The second group was healthy controls consisted of five dogs kept at Khartoum State, in the Police Dogs Directorate.


All dogs from group 1&2 were checked free from *Leishmania donovani*. They were vaccinated with routine vaccines (Distemper, canine adenovirus type 2, canine parvovirus and rabies). Each dog was labeled and had ID card before sending to the endemic area in order to record all the observations. Physical examination was obtained by veterinary personnel. Dogs were treated with antihelminthic as well as Ivomec (for ticks, mites and other external parasites). The field study was continued for more than 2 years (June 2006 – February 2009).

Trips for sampling and data collection were regulated every (1-2) months.
2.3. Samples collection:
Venous blood in plain vacutainer tubes, blood spots on filter papers (Whatman chromatography No3) (W&R Balston Ltd, England), lymph nodes, bone marrow aspirates, from dogs, were collected for performance of the following tests:
1- Parasitological (Direct microscopy) and culture on NNN media.
2- Serological (immunological) tests, DAT.
3- Molecular – biological (PCR).
Plate (5-a): The working team.

Plate (5-b): Two technicians taking Bone marrow & lymph node aspirate.

Plate (5-c): taking blood from a dog.

Plate (5-d): taking bone marrow sample
*D.N. = Disposable needle* for bone marrow aspiration.

Plates (5): Sampling of dogs for different diagnostic techniques.
2.4. **Body weight and clinical examination:**
The body wt. was recorded in kg every trip (1-2 months). Clinical examination for evaluation of clinical parameters such as the size of popliteal or supra – scapular, lymph nodes, claws (nails) and any other skin or body changes.

2.5. **Parasitological methods:**
The diagnosis of venous blood, lymph node and bone marrow aspirates of dogs from group 1 and 2 was carried out using direct microscopy examination of Geimsa stained smears and in vitro culture.

2.5.1. **Direct Microscopy:**
**A-Thin smears** from peripheral blood were prepared on glass slides, allowed to air dry, fixed with 100% methanol and labeled with dog no. and date. Stained with Geimsa and then examined under oil immersion (100-x magnification) for the presence of amastigotes.

**B-Bone – marrow aspirate:**
Under local anaesthetic (2ml Lidocaine+1ml Ketamine hydrochloride), about 1ml of bone marrow was aspirated from the iliac crest with a sterile bone marrow aspirate needle, and a 10 ml syringe. Thin smears were made, allowed to dry, fixed with 100% methanol, stained with Geimsa and then examined under oil immersion 100-x magnification (WHO, 1996).
c- Lymph node aspirate:
Popliteal or supra – scapular lymph node was grasped between thumb and fingers, a 21 gauge needle attached to a 5 ml syringe introduced into the lymph node, the lymph node pressed gently several times and then removed. Thin smears were made from lymph node aspirate as described in b- above and then examined under the microscope for the presence of Leishman Donovan bodies (L. D). (Evans, 1980, WHO, 1996).

2.5.2. In vitro culture:
Bone marrow and lymph node aspirates collected from dogs were "cultured" into NNN media prepared in Institute of Endemic Diseases, University of Khartoum. The media were supplemented with defibrinated inactivated rabbit blood and antibiotics (5mg Gentamycine in 10ml blood). The inoculated culture media were then transferred as soon as possible into an Ice bag container to the laboratory of the Institute of Endemic Diseases, where they were incubated at 24°C for 2 months, in a cool incubator. Each culture was checked under aseptic conditions 2wks later to detect parasite growth. The examination was continued for the remaining period by examining drops of culture fluid on glass slides under microscope for motile promastigotes.
2.6. **Serological tests:**
The testing of samples was performed in Khartoum University. Laboratory of *Leishmania* Research – Faculty of Science using the direct agglutination test.

2.6. **Direct agglutination test (DAT):**
DAT antigen was obtained from Department of Medical Microbiology – Faculty of Medicine. Ahfad University, prepared as described by (El-Harith et al., 1995).
The DAT was performed as described by (El-Harith *et al.*, 1989). Discs corresponding to 5µl of blood were punched out of filter papers, placed in a v-shaped micro titer plates and eluted with 0.9 %( wt/vol.) Physiological saline (200 µl each).
The plates were left at ambient temperature overnight. A fresh diluent prepared from physiological saline and 0.2% gelatin , heated to 56°C in water bath; after cooling (0.2m 2- Mercapto – ethanol was added . 75 µl diluent were pipetted into the v-shaped micro titer plates and 50 µl of eluate were added to each well , followed by 50 µl of antigen. The plate was covered with a plastic film, tilted gently for 1minute and incubated for 18hrs. at room temperature.
Negative control wells (Antigen+ diluent only) on each plate, and known positive and negative controls were tested.
After 18 hrs. incubation, the DAT was read independently by 2 individuals, by placing the micro titer plate on a white back ground.
The test was read by locating the first sharp button in the row, which is identical with the one in the control well, the preceding dilution was then considered the highest positive dilution or the titer of the test serum.

Titers of ≥ 1:1600 were considered positive.

2.7. Molecular Biological Methods:

2.7.1. DNA extraction:

DNA was isolated as described by (Osman, et al., 1997) from blood spots collected on whatman No. 3 filter papers, bone marrow and lymph node aspirates in lyses buffer.

Bone marrow and lymph node aspirates in lyses buffer (50mM NaCl, 50mM Tris – HCl [PH 7.4] ,10mM EDETA; 1%[vol/vol]Triton X - 100, 200 µg of proteinase k per ml ), were incubated overnight at 56°C. The samples were then subjected to phenol – chloroform extraction, precipitated with ethanol, and re- dissolved in 50µl of TE buffer (10mM Tris –HCl, 1mM EDTA [PH7.5]).

Blood spots on filter papers were placed between two sheets of clean paper and holes were punched out with a paper puncher. After each sample was obtained, a clean sheet of paper was punched 10-12 times in order to prevent DNA contamination from one sample to the next. Two punches (containing approximately 15µl of blood were placed in 250µl of lyses buffer. The DNA was extracted as described above.
2.7.2. PCR amplification:
The PCR amplification method described by (Meredith et al., 1993) was used, with minor modifications. 5µl of isolated DNA were added to 20µl of PCR mixture containing 2.5µl of 10X PCR buffer, 1µl of deoxynucleotides triphosphate, 0.2µl of taq DNA polymerase (Cinna Gen CO.) and 2p mol of each primer pair. The variable factors included the MgCl₂ concentration and the primer annealing temperature. The reactions were cycled in (TECHNE, TC-412) thermal cycler. The following conditions were used: The samples were pre- incubated at 110°C for 4min., followed by initial denaturation at 95°C for 5min. and 35 cycles consisting of denaturation at 94°C for 1min, and variable annealing temperature according to the primer set for 1 min and elongation at 72°C for 1 min., (table 1) followed by final extension at 72°C for 10 minutes.
A negative control tube that received 5µl d. d H₂O instead of DNA included in each test to detect any carry over contamination and a positive control tube was included in each test for the detection of PCR inhibition.
The reaction products were visualized under UV lights after electrophoresis of 8µl of the reaction solution in a 1-1.5 percentage agarose gel and a 100– b p. DNA ladder (Vivantis®) was used as a marker.
2.8. Questionnaires:
A questionnaire was designed to know the association between keeping domestic animals inside houses and the infection with visceral leishmaniasis in the endemic study area. The questionnaire included questions about the number of the householders, tribes, present and previous cases of VL, the domestic animals they were kept and their numbers. Another questionnaire filled during a home-to-home survey carried out December 2008-February 2009 to determine the prevalence of VL infection among local dogs of the study area. This questionnaire designed to gather basic information for each dog. (Appendix 1 – a & b).
Both questionnaires were designed to evaluate their ownership and keeping in-door contributing to risk factors of visceral leishmaniasis.

2.9. Statistical analysis:
The data were analyzed using Statistical Package for Social Science (SPSS) version 13.
Student T. test was used to compare the means of the body weight between control and naturally infected dogs within different periods of study. The Chi-Square test was used for the analysis of the questionnaire data of risk factors of VL infection.
### Table (1): Main characteristics of the four PCR methods used in the study:

<table>
<thead>
<tr>
<th></th>
<th>Pia1-Pia2</th>
<th>Pia3-Pia4</th>
<th>DB8-AJS3</th>
<th>RV1-RV2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR DNA target</strong></td>
<td>Repetitive sequence of <em>Leishmania infantum</em> genome</td>
<td>Repetitive sequence of <em>Leishmania infantum</em> genome</td>
<td>kDNA minicircle (10,000 copies)</td>
<td>kDNA minicircle (10,000 copies)</td>
</tr>
<tr>
<td><strong>Product size (bp)</strong></td>
<td>100 bp</td>
<td>120 bp</td>
<td>809 bp, 700 bp, 400 bp</td>
<td>145 bp</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td><em>L. donovani</em> sensu lato</td>
<td><em>L. donovani</em> sensu lato</td>
<td><em>Leishmania</em> spp.</td>
<td><em>L. donovani</em> sensu lato</td>
</tr>
<tr>
<td><strong>Primer sequences</strong></td>
<td>5’ACGAGGTCAGCTCCACTCC3’ 5’CTGCAACGCCTGTGCTACG3’</td>
<td>5’CGGCTTCGCACCATTGCGTG3’ 5’ACATCCCTGCCCACATACGC3’</td>
<td>DB8: 5’CCAGTTTCCCGCCCCCG GAG3’ AJS3: 5’GGGGTTGGTGTAAATAGGG3’</td>
<td>5’CTTTTCTGGTCCGCGGTTAG G3’ 5’CCACCTGGCCTATTTTACACC3’</td>
</tr>
<tr>
<td><strong>Mg Cl₂ concn.</strong></td>
<td>3 µl</td>
<td>1.5 µl</td>
<td>4 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td><strong>Annealing temp</strong></td>
<td>64°C</td>
<td>63°C</td>
<td>60°C</td>
<td>59°C</td>
</tr>
</tbody>
</table>
CHAPTER THREE

RESULTS

In this study, 35 dogs (*Canis familiaris*) from Khartoum State (non-endemic area) transferred to endemic area as natural infection test group.

Five dogs were kept as non-endemic healthy controls in Khartoum State.

Twenty-five local dogs were sampled during a home-to-home survey.

Almost 17 out of 35 dogs (group 1) died or lost during the course of the study for different reasons, (nine died because of CVL, five dogs were lost & 3 died for unknown reasons).

The results of a 17 experimentally infected dogs are available as part of the project "Leishmaniasis in eastern Sudan: Studies on Reservoir hosts and vectors" were also used for comparison.

3.1. Clinical signs:

Most of the remaining dogs showed clinical signs throughout the course of the study (18/20=90%). The clinical manifestations differ in their severity and duration between different dogs. The first clinical sign of disease was, diarrhea showed by two dogs (no. 30 & 34) three months after transfer to endemic area.

According to their clinical conditions dogs were divided into asymptomatic only one dog (no. 41) =5%, oligosymptomatic (1 or 2
symptoms; 12 dogs=60%); and polysymptomatic (more than two symptoms; seven dogs=35%).

As table (2) shows all dogs had one or more of the following clinical manifestations: diarrhea, long nails, enlarged lymph nodes, poor appetite, loss of weight, loss of hair, skin ulcers, alopecia, cough (pneumonia) and conjunctivitis.

Local dogs from endemic area, which were screened for clinical signs of visceral leishmaniasis 84% (21 of 25 dogs), showed no clinical signs of disease (asymptomatic). Of the symptomatic dogs, 12 % (3/25) showed long nails and only 4 % (1/25) showed enlargement of lymph nodes.
Table No. (2): Major clinical signs of CVL in naturally infected dogs compared to experimentally infected dogs Group in Khartoum.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Clinical signs</th>
<th>No. of dogs</th>
<th>% of dogs</th>
<th>% Experimentally Infected dogs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local dogs</td>
<td>1. Long nails</td>
<td>3</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Enlarged lymph nodes</td>
<td>1</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Asymptomatic</td>
<td>21</td>
<td>84%</td>
<td></td>
</tr>
<tr>
<td>L. donovani Natural infection test group 35 dogs</td>
<td>1. Long nails</td>
<td>11</td>
<td>55%</td>
<td>58.8%</td>
</tr>
<tr>
<td></td>
<td>2. Enlarged lymph node</td>
<td>10</td>
<td>50%</td>
<td>76.5%</td>
</tr>
<tr>
<td></td>
<td>3. Loss of appetite</td>
<td>6</td>
<td>30%</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>4. Diarrhea</td>
<td>2</td>
<td>10%</td>
<td>5.9%</td>
</tr>
<tr>
<td></td>
<td>5. Weight loss</td>
<td>6</td>
<td>30%</td>
<td>64.7%</td>
</tr>
<tr>
<td></td>
<td>6. Skin ulcers</td>
<td>2</td>
<td>10%</td>
<td>29.4%</td>
</tr>
<tr>
<td></td>
<td>7. Non ulcerated skin nodules</td>
<td>1</td>
<td>5%</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>8. Hair loss</td>
<td>4</td>
<td>20%</td>
<td>11.8%</td>
</tr>
<tr>
<td></td>
<td>9. Alopecia</td>
<td>2</td>
<td>10%</td>
<td>11.8%</td>
</tr>
<tr>
<td></td>
<td>10. Pneumonia</td>
<td>1</td>
<td>5%</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>11. Conjunctivitis</td>
<td>1</td>
<td>5%</td>
<td>5.9%</td>
</tr>
<tr>
<td></td>
<td>12. Asymptomatic</td>
<td>1</td>
<td>5%</td>
<td>11.7%</td>
</tr>
</tbody>
</table>

* Experimentally infected dogs (El-Sayed et al., 2009). (Nd = Not done.)
Plate (6-a): Non ulcerated skin nodules.

Plate (6-b): Loss of body weight.

Plate (6-c): Alopecia and loss of body weight.
Plate (6-d): Loss of hair and ulceration of skin.

Plate (6-e): Elongation of nails in infected dogs.

Plate (6-f): Ulceration of skin.
3.2. Body weight:

Table No. (3): Body weight changes throughout the study period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Weight (Mean± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>2006</td>
</tr>
<tr>
<td>Control dogs</td>
<td>6.8±3.63</td>
</tr>
<tr>
<td>Transferred dogs</td>
<td>4.92±3.59</td>
</tr>
<tr>
<td>p-value</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Figure (6): Comparison of body weight loss between the transferred dogs in the endemic area and control dogs.
Poor body weight gain in the first season & loss of weight started 10 months post introduction to endemic area increased in some animals by the end of the study.

Slight difference in body weight gain shown between control and transferred dogs.

3.3. Immunological results:

3.3. Direct agglutination test (DAT):

A titer of 1:1600 was considered as indicative of visceral leishmaniasis.

All control group (5) dogs had titers below 1: 800, which were considered as negative.

DAT results of dogs from *L. donovani* natural infection test group and local dogs from endemic area are shown in Table (4).
Table (4): DAT titers of the different animal groups.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Trip #</th>
<th>N0. of samples</th>
<th>≤1:800</th>
<th>1:1600</th>
<th>1:3200</th>
<th>1:6400</th>
<th>1:12800</th>
<th>1:25600</th>
<th>(+VE) DAT</th>
<th>(+VE) DAT%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leishmania donovani Infection test group 35 dogs</td>
<td>1</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>91.7%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>63.6%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>13</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>46.2%</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>11</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>9.1%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>75%</td>
</tr>
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<td></td>
<td>9</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>54.5%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>63.6%</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
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<td>11</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>18.2%</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>66.7%</td>
</tr>
<tr>
<td></td>
<td>15</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
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<td>83.3%</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>100%</td>
</tr>
<tr>
<td>Local dogs 25</td>
<td></td>
<td>25</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>24</td>
<td>96%</td>
</tr>
<tr>
<td>Control group 5</td>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>
Table (5): The DAT positive results throughout the study period

<table>
<thead>
<tr>
<th>Year</th>
<th>DAT +VE %</th>
</tr>
</thead>
<tbody>
<tr>
<td>At the start of the Study</td>
<td>0%</td>
</tr>
<tr>
<td>2006</td>
<td>100%</td>
</tr>
<tr>
<td>2007</td>
<td>92.9%</td>
</tr>
<tr>
<td>2008</td>
<td>100%</td>
</tr>
</tbody>
</table>

Figure (7): Shows variation of positive DAT percentage of transferred dogs through the study period.
For the local dogs sampled from the endemic area 96 % (24 out of 25) were found DAT positive with titers between (1:1600 – 1:25600), only one animal had a negative result with a titer of 1: 400 as shown by table (4).

*Leishmania donovani* natural infection test group of animals showed fluctuation in DAT titers, as table (4) shows. The number of DAT positive animals increased during the course of the study resulting in 75% (9 out of 12) positives at 2 weeks and 92.9% (13 out of 14) at 18 months post-introduction to the endemic area and reach 100%(6/6) by the end of the study.

As shown by figure (6), the percentage of the DAT positive dogs in the first season of the study (2006) was 100%, then dropped to 92.9 % (13/14) by 2007. In 2008 the percentage started to increase again reaching 100 %( six/6) with the highest titer of 1: 25600 by the end of the study in February 2009.

Number of DAT +ve dogs that were introduced to endemic area throughout the experimental period (6/06 – 2/09) excluding dogs that died or lost during the study (17dogs) =18/18(100%).
Plate (7): DAT plate showing the Detection of anti-*Leishmania* antibodies in (a) Transferred test group of dogs & (b) Local dogs.
3.4. Parasitological results:

3.4.1. Microscopical examination:

After 24 months, (2 years) post-introduction of dogs to the endemic area the first bone marrow and lymph node aspiration were taken. All blood samples, lymph node and bone marrow aspirates collected on glass slides for microscopy for the presence of *Leishmania* amastigotes were found negative.

3.4.2. Culture:

Only two samples of both lymph node, and bone marrow aspirates from the test group dogs 2/11 (18.2%) cultured into NNN media were +ve (dogs no. 2&29). The other samples either found –ve or did not grow because of microbial contamination.
### 3.5. The PCR results:

**Table (6):** PCR +ve Samples (percentage).

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample type</th>
<th>PCR Primer Pairs</th>
<th>Total +ve%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RV1- RV2</td>
<td>Pia1- Pia2</td>
</tr>
<tr>
<td>6/2008</td>
<td>B</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>6/9(66.7%)</td>
<td>6/9 (66.7%)</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>12/2008</td>
<td>B</td>
<td>2/6(33.3%)</td>
<td>2/6(33.3%)</td>
</tr>
<tr>
<td>2/2009</td>
<td>B</td>
<td>5/6(83.3%)</td>
<td>5/6(83.3%)</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>6/6(100%)</td>
<td>6/6(100%)</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>6/6(100%)</td>
<td>5/6(83.3%)</td>
</tr>
<tr>
<td>Local</td>
<td>B</td>
<td>14/25(56%)</td>
<td>19/25(76%)</td>
</tr>
<tr>
<td>dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B = Blood on filter paper, BM = Bone marrow, LN = Lymph node.
All the non-endemic controls (the five dogs kept in Khartoum) were PCR negative.

The first detection of parasite DNA was 24 months post-introduction to the endemic area in bone marrow samples only as shown by table (6), six out of 9(66.7%) and more than 2 years in peripheral blood and lymph node aspirates.

The number of positive animals increased during the course of the study resulting in 100% (6 out of 6) positive by the end of the study on blood spots, lymph node and bone marrow aspirates.

The four primers used showed different sensitivities, the kinetoplastic RV1-RV2 showed the highest sensitivity while the Deb8 –Ajs3 was the less sensitive one. For the other two genomic primer pairs, the Pia1-Pia2 showed the same sensitivity as the RV1-RV2 with the transferred dogs and the highest sensitivity with the asymptomatic local dogs, Pia3-Pia4 was less sensitive than Pia1-Pia2.

Ten out of eighteen (55.6%) of transferred dogs were found PCR positive throughout the study period as shown by table (7). Twenty-one out of Twenty five (84%) of the local dogs were found PCR +ve.
Figure (8-a) : Shows the results with specific kinetoplast primer pair RV1-RV2: Lane (1) Molecular weight marker (100bp), lane (2) +ve control reading band with size 145 bp, lane (3) -ve control, lane (4-16) transferred dogs’ (bone marrow& lymph node) samples (Feb /09).

Figure (8-b) : Shows the results with specific kinetoplast primer pair RV1-RV2: Lane (1) Molecular weight marker (100bp), lane 2) +ve control reading band with size 145 bp, lane (3) -ve control, lane (4-16) local dogs’ samples (Dec /08).
**Figure (nine-a):** DNA amplification results using Pia1-Pia2 genomic primer pair:
lane 1, ladder 100 bp. as a DNA molecular size marker, lane 2 +ve control,
lane 3 –ve control. Lanes, (4-16) transferred dogs’ blood samples (Feb/09).

**Figure (9-b):** DNA amplification results using Pia1-Pia2 genomic primer pair:
lane 1, ladder 100 bp. as a DNA molecular size marker, lane 2 +ve control from
*L. donovani*, lane 3 –ve control. Lanes, (4-16) local dogs’ blood samples(Dec/08).
**Figure (10-a):** DNA amplification results using Pia3-Pia4 genomic primer pair:
Lane 1, ladder 100 bp. as a DNA molecular size marker, lane 2 +ve control from *L. donovani*, lane 3 –ve control. Lanes, (4-16) transferred dogs’ lymph node & bone marrow samples (Feb/09).

**Figure (10-b):** DNA amplification results using Pia3-Pia4 genomic primer pair:
Lane 1, ladder 100 bp. as a DNA molecular size marker, lane 2 +ve control from *L. donovani*, lane 3 –ve control. Lanes, (4-16) Local dogs’ blood samples (Dec/08).
**Figure (11-a):** DNA amplification results using AJS3-Deb8 kineto plast primer pair: Lane 1, ladder 100 bp. as a DNA molecular size marker, lane 2 +ve control from *L. donovani* patient, lane 3 –ve control. Lanes, (4-16) Transferred dogs’ blood, lymph node & bone marrow samples (Feb/09).

**Figure (11-b):** DNA amplification results using AJS3-Deb8 kinetoplast primer pair: Lane 1, ladder 100 bp. as a DNA molecular size marker, lane 2 +ve control from *L. donovani* patient, lane 3 –ve control. Lanes, (4-16) Local dogs’ blood samples (Dec/08).
Table (7): Comparison between Cultures, DAT& PCR Results

<table>
<thead>
<tr>
<th>Animal group</th>
<th>+ve (Culture)</th>
<th>+ve (DAT) ≥1600</th>
<th>+ve PCR</th>
<th>+ve (DAT&amp;PCR)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferred dogs</td>
<td>2/11 (18.2%)</td>
<td>18/18 (100%)</td>
<td>10/18  (55.6%)</td>
<td>10/18  (55.6%)</td>
<td>18</td>
</tr>
<tr>
<td>Local dogs</td>
<td>N. D</td>
<td>24(96%)</td>
<td>21(84%)</td>
<td>21(84%)</td>
<td>25</td>
</tr>
</tbody>
</table>

N. D= not done

3.6. The Results of the Questionnaires:

The statistical data analysis shows that there was a strong association between VL infection and keeping domestic animals inside or around human dwellings. (Figure 12) and appendix (2).

In addition, the data collected about local dogs showed a significant association between dog ownership and visceral leishmaniasis infection. (Figure 13) & appendix (3).
Figure (12): The significant association (P ≤ 0.05) of keeping domestic animals indoor with the VL infection.
Figure (13): The significant association of the presence of dogs indoors and the infection with VL \( P \leq 0.05 \).
CHAPTER FOUR
DISCUSSION

The increasing domestication of the transmission cycle of some Leishmania species has caused dramatic changes in the epidemiology of leishmaniases, such as changes in transmission patterns due to the adaptation of these parasites to new vectors and hosts. (Dantas-Torres, 2007).

It is well known that the dog plays an important role in the zoonotic cycle of transmission of L. (L.) infantum (Dantas-Torres, 2007).

Cases of natural infection of dogs by L. arabica (Peters et al., 1986), L. (L.) major (Elbihari et al., 1987; Morsy et al., 1987), Leishmania (Leishmania) tropica, (Dereure et al., 1991; Guessous-Idrissi et al., 1997), L. (V.) peruviana (Llanos-Cuentas et al., 1999), and Leishmania (Leishmania) donovani (Dereure et al., 2003) have been documented.

To our knowledge, this is the first investigation of this type carried out in Sudan by introducing dogs from non endemic area to VL endemic area to elucidate the natural L. donovani infection in dogs and then to determine their role in transmission of visceral leishmaniasis as reservoir hosts.
In this study, most of the clinical features showed by the introduced test group of dogs were similar to those described by Ferrer et al., (1988).

In the present study, the overall typical CVL symptomatic features were observed in 90% of the transferred dogs (naturally infected dogs). This percentage is almost similar to that reported by Elsayed et al, (2009). However, looking closer into some of these features a marked variation in the prevalence as well as the degree of severity can be observed.

All these differences in clinical aspects may be due to the high dose of cultured promastigotes from the stationary phase inoculated intravenously in experimentally infected dogs and inoculums used here do not include saliva of the insect vector (natural infection).

One of the main objectives of the present study was also to compare the clinical features observed on the introduced dogs to those features on the locally reared dogs within the endemic area. Only 16 percentage of the local dogs showed symptomatic CVL features. On the other hand, the majority (84%) were asymptomatic. This may be explained within the context of the fact that immunological responses in which the introduced dogs appears to be more susceptible and immune suppressed compared to local dogs. These observations have also been demonstrated in CVL endemic areas where high percentages of dogs have had contact with the parasite as established by the presence of
specific antibodies or of specific cellular immune response, but many have no signs of disease (resistant) (Ferrer, 1999). The same author suggested that mongrel dogs and some Spanish autochthonous breeds are more resistant than imported breeds. Cellular responses against *Leishmania* have been detected in dogs and this type of immunity appears to be associated with resistance to infection. Cabral *et al.*, (1998) first detected *Leishmania*-specific lymphocyte proliferation in asymptomatic dogs from an endemic region. In addition, the existence of cellular responses in infected dogs was confirmed by Pinelli who found specific lymphocyte proliferation in experimentally infected asymptomatic animals but not in symptomatic dogs (Pinelli *et al.*, 1994).

In areas where zoonotic visceral leishmaniasis is endemic, the prevalence of *L. (L.) infantum* infection in dogs is often high, with a large proportion of asymptomatic ones (Dantas-Torres *et al.*, 2006).

Loss of body weight or failure to put on weight in test group of introduced dogs could be attributed mainly to anorexia and diarrhea in some animals.

Loss of weight was apparent ten months post introduction to endemic area; this result is in contrast to that obtained by El-Sayed *et al.*, (2009) who observed loss of weight 8 weeks post infection. This shorter period might be attributed to the severity of infection in case of the experimentally infected group. No significant difference between the
transferred and control group of dogs in body weight changes; this result was in agreement with that obtained by El-Sayed et al., (2009). Few investigators have proposed possible causes for the weight loss that is generally associated with leishmaniasis. Local infiltration of infected macrophages in the mucosa of the gut has been proposed Adler et al., (1934).

Serological examinations of the dogs for the prevalence of anti-Leishmania antibodies, excluding dogs that died or were lost during the study (17 dogs), showed that 18/18 (100%) of the introduced dogs and 24/25 (96%) of the tested local dogs were DAT positive (The cutoff dilution was set at 1:1600). These results were much higher compared to those obtained by Hassan et al., (2009) who found that only 6.9% and 51.7% of dogs were positive by DAT and IFAT, respectively in villages of Rahad River eastern Sudan. The sero-positivity by DAT in dogs was 100%, 92.9% and 100% in 2006, 2007 and 2008, respectively. These results show the relative stability of sero-positivity in the test group introduced dogs. These results are also higher than results reported by Dereure et al., (2003) in an endemic area eastern Sudan Barbar el–Fugara (that is the same area in which the present investigation was conducted), who found , 43–74% of dogs were sero- positive by IFAT.
Also in a case-control study to evaluate risk factors for visceral leishmaniasis during an epidemic in Amhara Region, Ethiopia, Bashaye et al., (2009) found that the prevalence of infection among surveyed dogs in the outbreak villages was only 10.8% by IFAT, 3.8% by ELISA, and 2.8% by PCR.

The highest titer of 1: 25600 was reached by the end of the study in February 2009; this may be due to accumulation of antibodies because of repeated infection or Leishmania infection itself. The elevated titers (>1:320) obtained in 10 cases which had been diagnosed only on clinical grounds can be attributed with great certainty to Leishmania infection. (El– Harith, et al., 1989).

Studies on dogs either naturally or experimentally infected with L. infantum or L. chagasi have shown that 1) asymptomatic dogs can be infectious to sand flies; (Molina, et al., 1994; Alvar, et al., 1994; Vexenat, et al., 1993), 2) dogs become infectious to sand flies after a median period of about 200 days; 3) infectiousness may be associated with high antibody titers; (Molina, et al., 1994).

Microscopical examination of smears made from blood, lymph nodes and bone marrow aspirates were found negative. The same results were shown by (Hassan et al., 2009) and (El-Sayed et al., 2009). This low sensitivity of direct microscopy was demonstrated by many researchers in peripheral blood (Zijlstra et al., 1992; Osman, 1997) and
in bone marrow, 60% and about 30% in lymph node smears (Ferrer, 1999).

Two dogs out of eleven (18.2%) showed positive results in both bone marrow and lymph node aspirates cultures in NNN media. These results are lower than results obtained by Dereure et al., (2003) who found that $23/79 = (29\%)$ of lymph node, cultures were positive. This may be attributed to low parasite loads especially in early stages of canine leishmaniasis and microbial contamination of some cultures.

The first detection of parasite DNA was 24 months post-introduction to the endemic area on bone marrow samples and more than 24 months on peripheral blood and lymph node aspirates.
Six out of 9 (66.7%) animals tested were found positive in PCR on bone marrow samples only. This result showed that PCR on bone marrow samples was more sensitive than PCR on peripheral blood.

The same results were obtained by many researchers. In general, PCR is a more sensitive method for the detection of *Leishmania* in lymph node and especially bone marrow aspirates of VL patients than microscopy and is especially useful for the confirmation of cases of suspected VL, Schallig and Oskam, (2002). Bone marrow aspirates from parasitologically confirmed VL patients were always PCR positive in several studies (Mathis & Deplazes 1995; Andresen *et al.*, 1997).
It must be emphasized that if PCR on blood is negative, a PCR on lymph node and/or bone marrow material should be performed, because PCR on these materials is more often positive (Osman et al., 1997a).

In another study, comparison of PCR on bone marrow aspirates with microscopic examination and culture for diagnosis of VL in immune-compromised patients showed that PCR exhibited a higher sensitivity (82%) than microscopy (55%) and culture (55%) (Piarroux et al., 1994).

Although requiring an invasive procedure, BM biopsy is usually considered the sample of choice for a sensitive diagnosis of canine leishmaniasis, compared with less invasive materials such as peripheral blood (Oliva, et al., 2006).

The four primers used in this study showed different sensitivities, the kinetoplastic RV1-RV2 primer showed the highest sensitivity while the Deb8–Ajs3 was the less sensitive one. These results supported results obtained by Lachaud, et al., (2002b), who stated that, two methods targeting the highly repetitive kinetoplast DNA, interestingly, one method targeting kinetoplast DNA was less sensitive than genomic-DNA-based methods, showing that PCR sensitivity is not necessarily correlated to the degree of reiteration of the DNA target. Reithinger, et al., (2000) has also noted this with different kinetoplastic primer sets.
In this study the genomic -DNA-based method, Pia1-Pia2 showed the same sensitivity as the RV1-RV2 with the transferred dogs and the highest sensitivity with the asymptomatic local dogs. The same results were obtained by Saeed, (2009) in detection of *Leishmania donovani* in domestic and wild animals in Eastern Sudan. This may be related to natural infection in both studies.

In this study, a total of 10/18 (55.6%) of transferred dogs were found PCR positive throughout the study period. These results were in line with the results recorded by Oliva, *et al.*, (2006), who found that, the highest number of positives was found with the n-PCR examination of BM (54.5%) and did not match with the results obtained by Hassan *et al.*, (2009), who found that only two dogs out of 87 (2.3%) were PCR +ve by specific primer pairs.

These PCR results were much lower than those obtained by El-Sayed *et al.*, (2009) in an experimental infection of indigenous dogs, who found that 17 dogs out of 17 (100%) were PCR +ve by the same primer pairs.

These differences may be due to the following aspects: the experimental infection 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 does not include saliva of the insect vector as in natural infection. Also 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 as stated by Jacqueline Poot, (2006).

Twenty-one out of twenty five (84%) of the local dogs were found PCR +ve.

Comparing the results of DAT and PCR, only 10/18(55.6%) were found PCR positive, while all 18/18(100%) were found DAT positive in the remaining transferred dogs. 84% PCR positive and 96% DAT positive in the local dogs.

This is related to that PCR can detect active or current infection, whereas DAT cannot distinguish between current and past *Leishmania* infections (Zijlstra, *et al*., 1991).

As DNA persists in the body for only a short time (24 h), PCR positivity is a good indicator of current (or recent) infection (Prina, *et al*., 2007)

Increasing risk factors are making leishmaniasis a growing public health concern for many countries around the world (Desjeux, 2001). Many epidemiological studies based on household surveys to determine the risk factors that are contributing in the transmission of VL in endemic areas, have been carried out in Iran (Gavagani, *et al*., 2002), in Bangladesh (Bern, *et al*., 2005), Kenya (Kolaczinsky, *et al*., 2008), Ethiopia (Bashaye, *et al*., 2009), Nepal (Bhattarai *et al*., 2010).
Most of those studies associated the increase of VL with the decrease of socio-economic status of the house-holders, such as malnutrition, poor housing conditions, lack of preventive measures in the form of sanitation and bed nets, illiteracy, beside other factors such as, keeping domestic animals in-door or ownership of domestic dogs. In Sudan, similar surveys correlated risk factors of VL to environmental factors (Elnaiem, et al., 2003) or genetical factors (Mohammed, et al., 2003).

In an investigation of an outbreak in a community in eastern Sudan, a large proportion of villagers appeared to have been infected over a 4-year period. Risk factors for VL included ownership of dogs and cattle, younger age, and male gender, whereas the presence of a neem tree was protective. Ethnicity appeared to be another important predictor of risk. (Bucheton, et al., 2002). In Kenya Kolaczinski, et al., (2008) suggested the role of domestic animals in reduction of VL risk due to their attraction of the sand fly vectors away from human, in contrast Gavagani, et al., (2002), Bashaye, et al., (2009) and Bhattarai et al., (2010) showed that domestic animals including dogs increase the risk of VL infection.

During this study, a positive association between dog ownership and human VL was identified. Based on the risk factor analysis, a significant association was also observed between human infection and the presence of domestic mammals in or around human dwellings. Similar results were showed by Saeed, (2009).
Gavagani, *et al.*, (2002), showed that owning a dog is a significant risk factor for members of households. They also showed that human infection rates increased with dog density, and proposed that dog density affects local transmission rates to humans by increasing the prevalence of infection among sand flies, so increasing the entomologic inoculation rate.

A possible association was observed between human infection and the presence of dogs in or around human dwellings (Cunha, *et al.*, 1995). In univariate conditional logistic regression analyses, dog ownership, keeping cattle inside the house at night, report of indoor insecticide spraying, and increasing family size were associated with significantly higher risk of VL. (Bashaye *et al.*, 2009).

A positive association between dog ownership and human ACL was identified in two studies in Argentina (Estani, S. and others, unpublished data) and Costa Rica, (Rojas, *et al.*, 1994), respectively. Dogs are usually in or next to human houses, which favors the maintenance of the domestic transmission cycle of *L. (L.) infantum* (Dantas-Torres and Branda˜o- Filho, 2006). Not necessarily acting as reservoirs of infection, but attracting vectors to human dwellings, for example. (Dantas-Torres, 2007).
In eastern Sudan, two studies based on host feeding preference of Phlebotomus orientalis on different animals showed clear preference of the vector to the domestic dog. (Saeed, 2009; Hassan et al., 2009).
Conclusion and Recommendations

The high seroprevalence (96%) in the local dogs and (100%) of the transferred test group of dogs, high PCR results (84%) and (55.6%) respectively, (18.2%) of transferred dogs positive in culture and clinical manifestations of canine visceral leishmaniasis demonstrated by this study have led us to assume that the dog plays an important role in the transmission cycle and may act as a reservoir host of kala-azar in this endemic area of VL in eastern Sudan.

The data collected based on risk factors showed significant association between dog ownership, keeping domestic animals in door or close to householders and VL infections. This increases the assumption that these domestic animals including the dogs may be involved in the transmission of VL, either by harbouring the Leishmania parasite, or by attraction of sand flies to the human dwellings.

Further investigations are needed on the vectors, characterization of the parasite strains in the sand flies, infected humans and dogs by the most advanced molecular methods for a better understanding of the Leishmania species and their epidemiological status in Sudan. Correct identification of the Leishmania reservoir host is very important for the design of control programs to cut the transmission
cycle of *Leishmania*, beside other control measures such as, improving housing conditions, environment sanitation as well as personal protective measures, such as the use of insecticide-treated bed nets, and to provide rapid diagnosis and treatment of the affected persons.
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www.wikipedia.com
APPENDIXES

APPENDIX (1): Questionnaires
(a) Keeping domestic animals inside houses.

<table>
<thead>
<tr>
<th>Number of householders infected by Kala-azar</th>
<th>No. of animals in home</th>
<th>Type of animals</th>
<th>Tripe</th>
<th>No. of householders</th>
<th>Name of householders head</th>
<th>Home no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>Previous</td>
<td>16</td>
<td>Cows+ donkeys + chicken+goat</td>
<td>Falatta</td>
<td>5</td>
<td>Mohamed Osman</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>17</td>
<td>Cows + donkeys + sheep</td>
<td>Beni - Amir</td>
<td>10</td>
<td>Ali Omer</td>
</tr>
</tbody>
</table>

Appendix (1): (b) dog ownership

<table>
<thead>
<tr>
<th>Number of householders infected by Kala-azar</th>
<th>Age of dog/years</th>
<th>Area / village</th>
<th>Tripe</th>
<th>Name of dog owner</th>
<th>Home no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>Previous</td>
<td>7</td>
<td>Barber el- fugara</td>
<td>Tama</td>
<td>Issa Osman</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4</td>
<td>Tabark- alla</td>
<td>Masaleet</td>
<td>Yahya Abbakar</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
APPENDIX (2): (i) Breeding animals inside houses * infection with kala-azar - Cross tabulation

<table>
<thead>
<tr>
<th></th>
<th>X33</th>
<th>Count</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>توجد إصابة</td>
<td>لا توجد إصابة</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>196</td>
<td>1</td>
</tr>
<tr>
<td>% within X22</td>
<td>99.5%</td>
<td>99.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within X33</td>
<td>87.1%</td>
<td>87.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>86.7%</td>
<td>86.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td></td>
<td>195</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>لا توجد</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>29</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>% within X22</td>
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<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within X33</td>
<td>12.9%</td>
<td>12.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
<td>12.9%</td>
<td>12.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>225</td>
<td>1</td>
</tr>
<tr>
<td>% within X22</td>
<td>99.6%</td>
<td>99.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% within X33</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Total</td>
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<td>100.0%</td>
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</tr>
</tbody>
</table>
(ii) Chi-Square Tests

<table>
<thead>
<tr>
<th>Exact Sig. (1-sided)</th>
<th>Exact Sig. (2-sided)</th>
<th>Asymp. Sig. (2-sided)</th>
<th>Df</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.700</td>
<td>1</td>
<td>.149</td>
<td>Pearson Chi-Square</td>
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<tr>
<td></td>
<td>1.000</td>
<td>1</td>
<td>.000</td>
<td>Continuity Correction</td>
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<tr>
<td></td>
<td>.599</td>
<td>1</td>
<td>.277</td>
<td>Likelihood Ratio</td>
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<tr>
<td>.871</td>
<td>1.000</td>
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<td></td>
<td>Fisher's Exact Test</td>
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<tr>
<td></td>
<td>.700</td>
<td>1</td>
<td>.148</td>
<td>Linear-by-Linear Association</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N of Valid Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>225</td>
</tr>
</tbody>
</table>

a  Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is .13.

APPENDIX (3): Keeping dogs inside houses * infection with kala-azar - Cross tabulation

<table>
<thead>
<tr>
<th>Total</th>
<th>X22</th>
<th>X11</th>
</tr>
</thead>
<tbody>
<tr>
<td>توجد اصابة</td>
<td>لا يوجد اصابة</td>
<td>Count</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>100.0%</td>
<td>4.0%</td>
<td>96.0% % within X11</td>
</tr>
<tr>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0% % within X22</td>
</tr>
<tr>
<td>100.0%</td>
<td>4.0%</td>
<td>96.0% % of Total</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>100.0%</td>
<td>4.0%</td>
<td>96.0% % within X11</td>
</tr>
<tr>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0% % within X22</td>
</tr>
<tr>
<td>100.0%</td>
<td>4.0%</td>
<td>96.0% % of Total</td>
</tr>
</tbody>
</table>