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Monitoring of Anti *Leishmania* Antibody Responses for Early Diagnosis and Prognosis of Visceral Leishmaniasis in Dinder National Park

By

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Abbreviations:

AVL: anthroponotic visceral leishmaniasis
CL: Cutaneous leishmaniasis
CSA: crude soluble antigen
DAT: Direct agglutination test
DNA: Deoxy ribonucleic acid
DTH: Delayed-type hypersensitivity
ELISA: Enzyme linked Immuno Sorbant Assay
IFAT: Indirect fluorescence antibody test
Igs: Immunoglobulins
IL: Interleukin
IFN: Interferon
kDNA: Kinetoplast DNA
MCL: Mucocutaneous leishmaniasis
ML: Mucosal leishmaniasis
NO: Nitric oxide
PBS: Phosphate buffer saline
PCR: Polymerase chain reaction
PKDL: Post kala-azar dermal leishmaniasis
Th: T helper cells
TNF: Tumor necrosis factor
VL: Visceral leishmaniasis
ZVL: zoonotic visceral leishmaniasis
Abstract

Introduction: Visceral leishmaniasis is a serious health problem endemic in many regions of Sudan. The outcome of *leishmania* infection depends on the infecting *leishmania* species and the host immune response. It is known that the majority of *leishmania* infected hosts remain asymptomatic. There is a little information available on the seroconversion and the outcome.

Materials and methods: A longitudinal prospective study was conducted for 12 months in Dinder National Park in central Sudan. The area is known to be highly endemic for VL. Wild Animal Guards in the Park were consented and recruited to the study. A total of 110 guards were tested in two surveys for detection of anti *leishmania* antibodies IgG, IgM, IgG1, IgG2, IgG3 and IgG4 were measured in serum sample collected from the participants using DAT and ELISA. Attempts to detect circulating *leishmania* DNA were done using PCR amplification of *leishmania* kDNA. Paired sample were obtained from 31 participants.

Results: Forty-six guards were IgG positive during the period of the study using DAT while eighty-five were positive using ELISA. Two guards had antileishmania IgM detected by ELISA. Of the participants who were positive in IgG had dominate IgG subclasses Sixteen had IgG1 ELISA, 38 had IgG2 ELISA, 12 had IgG3 and 3 had IgG4 ELISA.

Conclusion: The majority of individuals exposed to *leishmania* infection develop asymptomatic infection. The infection induces IgM and IgG antibody responses. The role of the induced humoral response in protection and susceptibility against
*leishmania* is not well defined. Further studies needed to investigate the evolution of antibody responses in *leishmania.*
Arabic Abstract

المستخلص

مقدمة: يعتبر داء انفاث النسيم مشكلة صحية خطيرة في مناطق عدد من البلدان. وعديوان انفاث النسيم تعتد على نوع الطفيل المسبب والاستجابة المناعية للعلاق. إذ تؤدي إلى مجيء لمجموعة من الأشخاص المصابين بانفاث النسيم لا تظهر عليهم أعراض المرض وهناك القليل من المعلومات المتوفرة عن التشخيص والتحول المصل وتدبير معالجة المرض عند الأشخاص الأصحاء.

المؤلفات والطريقة: أجريت دراسة طويلة المدى لمدة 12 شهراً في طواف الدندو الوريدي في وسط السودان والموضوع بناءً على دراسات العشاق الوراثي في الدراسة وذلك بعد موافقتهم وجرى اختبار ما مجموعه 110 من حساس العوائد البرية في مسح أنفاث النسيم المصابون G، G1، G2، G3، G4 المباشراً واختبار الالبوز. عدة محاولات تم للكشف عن الحمض النووي للنسيم كما استخدم تفاعل البلوريمز التسلسلي وذلك لمضاعفة الحمض النووي الخاص با لكينو تيرينس. هناك واحد وثلاثون من المشاركون تم إخضاعهم منهم منتظم.

النتائج: سنة وأربعون من المشاركون في الدراسة كان لديهم اختبار إيجابي للغلبولين المناعي G في المراحل الأولى والثانية وذلك باستخدام اختبار التراص الدموي المباشراً وخمسمائة والتسعون لديهم اختبار إيجابي باستخدام الالبوز واثنان لديهم اختبار موجب للغلبولين المناعي M با استخدام الالبوز 16 لديهم اختبار موجب للغلبولين المناعي G با استخدام الالبوز 38 لديهم اختبار موجب للغلبولين المناعي G1 با استخدام الالبوز 12 لديهم اختبار موجب للغلبولين المناعي G2 با استخدام الالبوز 25 لدىهم اختبار موجب للغلبولين المناعي G3 و 3 لديهم اختبار موجب للغلبولين المناعي G4 با استخدام الالبوز.

الخلاصة: غالبية الافراد الذين تعرضو لطيفان انفاث النسيم ليس لديهم أعراض المرض. الإصابة تحت انتاج الغلبولين المناعي G والغلبولين المناعي M. دور الاستجابة المناعية الخلوية في الوقاية من المرض أو القابلية للإصابة بالموضوع لا زال غير واضح. المزيد من الدراسات يجب أن تجري من أجل معرفة تطور الاجسام المضادة لطيفان انفاث النسيم.
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Chapter One

INTRODUCTION AND LITERATURE REVIEW

1.1. Leishmaniasis:

Leishmaniasis is a major vector-borne disease caused by intramacrophage protozoa of the genus *Leishmania* parasites infecting several mammalian species including humans. Leishmaniasis is transmitted by the bite of phlebotomine sand flies (Bañuls *et al.*, 2007). The disease; leishmaniasis is endemic in areas of the tropics, subtropics and southern Europe (Dujardin *et al.*, 2008).

It is endemic in 88 countries Twelve (12) million people are infected, 350 millions are considered at high risk of infection and 1.5 to 2 million new cases are thought to occur annually (WHO, 2000).

The geographical distribution of leishmaniasis is limited by the distribution of the vector. Ninety percent (90%) of the new Visceral Leishmaniasis (VL) cases occur in five countries: Bangladesh, Brazil, India, Nepal and Sudan. Ninety (90%) of the Mucocutaneous Leishmaniasis (MCL) cases occur in Bolivia, Brazil and Peru, while 90% of the Cutaneous Leishmaniasis (CL) cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (WHO, 2000). Currently, leishmaniasis has a wider geographical distribution pattern than before and it is considered to be a growing public health concern for several countries. The increase in leishmaniasis’ worldwide incidence is mainly attributed to the increase of several risk factors that are clearly man made and include massive migration, deforestation, urbanization, immunosuppression, malnutrition and treatment failure (Desjeux, 2001).
1.2 Clinical forms of Leishmaniasis:

Leishmaniasis can be classified into four major clinical forms according to the severity: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL) and Post-kala-azar dermal leishmaniasis (PKDL) (Grimaldi et al; 1993).

1.2.1 Cutaneous leishmaniasis (CL):

Cutaneous leishmaniasis (CL) involves ulcerative lesions of the skin, which in general are self-limiting. It can be caused by several pathogenic Leishmania strains. Cutaneous leishmaniasis (oriental sore) in Sudan is caused by Leishmania major zymodeme LON1 (El Safi et al, 1991) and by Leishmania donovani (MON-82) (Elamin et al, 2008). Parasite is transmitted by the sandfly vector Phlebotomus papatasi (Kirk and Lewis, 1955).

1.2.2 Mucocutaneous leishmaniasis (MCL):

Mucocutaneous leishmaniasis (MCL) causes destructive lesions, especially of the nasopharyngeal mucosa and cartilages. Ninety percent of all cases of MCL occur in Bolivia, Brazil and Peru (WHO, 2000). In the vast majority of cases it is associated with parasites that belong to the Leishmania brasiliensis complex in the New World (Grimaldi et al; 1993.). In Sudan Mucosal leishmaniasis (ML) is caused by Leishmania donovani, Leishmania major and L.archibaldi (Ghalib et al, 1992 Lainson & Shaw et al, 1987, Oskam et al, 1998 ). El Hassan & Zijlstra (2001) characterized one isolate from Sudanese ML by isoenzymes and it was characterized as L.donovani zymodeme MON-18.
1.2.3 Visceral Leishmaniasis (VL):
Visceral leishmaniasis (VL) is a severe systemic disease characterized by involvement of the lymphoid organs and high rates of fatality in symptomatic patients. It is generally caused by parasites belonging to the *Leishmania donovani* complex, which are endemic in both the Old World (*L. donovani* and *L. infantum*) and the New World (*L. chagasi*) (Grimaldi, *et al.*, 1993). Clinically and epidemiologically there are two main forms: (1) zoonotic visceral leishmaniasis (ZVL), which affects mainly young children and has the domestic dog as its principal reservoir and (2) anthroponotic visceral leishmaniasis (AVL), which affects people of all ages, and transmitted from human to human via infectious sand fly bites. There are an estimated 0.5 million new cases of visceral leishmaniasis per year, concentrated in India, Nepal, Bangladesh, Sudan and Brazil, though this is likely to be an underestimate (Bern *et al* 2008, Reithinger, 2008). VL is also marked by high levels of leishmania-specific antibodies (Singh *et al.*, 1995) which appear soon after infection and before the development of cellular immunologic abnormalities, while the antibody titer in kala-azar have been exploited for specific diagnosis, their role in resolution of disease and protective immunity is largely unknown. It is, however, evident that resistance in a large population of individuals residing in areas of endemcity is detectable only by the development of specific antibodies and or T-cell response to leishmanial antigens (Kurtzhals *et al.*, 1992).
1.2.4 Post-kala-azar dermal leishmaniasis (PKDL):

Post-kala-azar dermal leishmaniasis (PKDL) is a dermal complication of kala-azar (visceral leishmaniasis). It is characterized by development of macules, papules or nodules following treatment of visceral leishmaniasis (El Hassan et al, 1992). Although it may happen without previous history of VL (Roustan et al, 1998). All members of L.donovani complex were reported to cause PKDL in addition to L.brazilensis in the new world (El Hassan et al, 2001).

In a prospective study in a village in the endemic area for kala-azar in the Sudan, 105 of 183 (57%) kala-azar patients developed PKDL. There was a significantly higher PKDL rate (69%) in those who received inadequate and irregular treatment with antimony (Zijlstra et al, 2000).

1.3 Leishmaniasis in the Sudan:

Leishmaniasis, especially visceral leishmaniasis (VL) is 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. Other smaller foci have also been described in 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 deaths (Sati et al, 1958). An outbreak of kala-azar was reported in Khartoum among displaced people (de Beer et al, 1990). Also, epidemics have occurred in recent years in war zones of southern Sudan where about 100,000 people died of leishmaniasis since 1984 (Seaman et al,
Transmission dynamics have not been elucidated fully; heavy transmission in relatively scarcely populated areas such as Dinder national park suggested zoonotic transmission whereas the large numbers of patients with post kala-azar dermal leishmaniasis (PKDL) in heavily affected villages may indicate a human reservoir and anthroponotic transmission. (El Hassan & Zijlstra, 2001).

*P. orientalis* is the only known proven vector of kala-azar in the Sudan (Hoogstraal and Heyneman, 1969; Elnaiem *et al.*, 1997). However, in the Kapoeta area in South Sudan, where *P. orientalis* is not known to be present *P. martini* may be the main vector of VL (Miniter *et al.*, 1962).

**1.4 The vectors:**

The vector of various clinical forms of leishmaniasis belong to Order: Diptera; Class: Insecta; Family: Psychodidae; and Phylum: Arthropoda. (Kalra and Bang, 1988). The parasite is transmitted by the bite of infected female sandflies: *Phlebotomus* in the Old World and *Lutzomyia* in the New World (central and south America). Morphologically they resemble very closely with each other. The name ‘sand fly’ can be confusing as this name is sometimes used for other species as well. Only 10% of the approximately 600 known species of sand flies are vectors (Kalra and Bang, 1988) and only 30 of these are known to transmit *leishmania* parasites, these include *P. argentipes* on the Indian sub-continent, *P. martini* and *P. orientalis* in Africa and the Mediterranean basin, *P. chinensis* and *P. alexandri* in china. In the new world *Lutzomyia logipalpis* is the only known vector (WHO, 1991).
*leishmania* is transmitted mainly by the blood-sucking females insects, which are 2-3 mm long and are found throughout the tropical and temperate parts of the world. The sand fly larvae require organic matter, heat and humidity for development and so are commonly found in house-hold rubbish, bark of old trees, burrows of old trees and in cracks in house walls. (Lewis and Ward, 1987).

**1.5 Reservoir hosts:**

Two cycles of *Leishmania* transmission were identified. The zoonotic cycle include infection of animals’ reservoir while in the anthroponotic cycle patients serve as source of parasites. Several animal species were incriminated as reservoirs including canine and rodents (WHO, 1991).

Wild and domesticated animals and humans can act as reservoirs for *Leishmania*. Small mammals serve as reservoirs for several *Leishmania* species and play a major role in the epidemiology of the disease. The importance of rodents in the epidemiology of the Leishmaniasis was demonstrated by (Hertig et al, 1957).

In anthroponotic visceral Leishmaniasis due to *L.d.donovani* as in India and possibly Sudan, man is considered as the principal reservoir host. In those cases asymptomatic carriers and PKDL patients are possible source of infection for sandflies (WHO, 1991).

**1.6 Life cycle of Leishmania parasite:**

*Leishmania* parasites’ life cycle is complex (figure 1). *Leishmania* has two basic life cycle stages: an extracellular stage within the invertebrate host (phlebotomine sand fly) promastigotes and an intracellular stage within a vertebrate host amastigotes (Koutis, 2007). *Leishmania* life cycle begins when an infected
female sand fly takes a blood meal from the vertebrate host. During the blood meal intake, the sand fly introduces its mouthparts into the skin tearing tissues and the salivary gland content is injected together with Leishmania promastigotes into the host’s skin (Titus et al., 1988; Andrade et al., 2007). The promastigotes are long, flagellate and extracellular.

The promastigotes are then phagocytosed by the host’s macrophages and consequently the parasite evolves into amastigote forms – spherical, intracellular forms without flagellum that reproduce by binary fission. The multiplication of the parasites occurs inside the macrophages, which are their main targets. The macrophage lyses and the cycle continue when other hosts’ phagocytes are being infected (Baños et al., 2007).
Figure (1): The life cycle of *leishmania* parasites National Institute of Allergy and Infectious Diseases (NIAID). (www.niaid.nih.gov).
1.7 Diagnosis of the *Leishmania* infections:

The diagnosis of *Leishmania* infections usually relies on direct examination of smears from affected tissues or organs after Giemsa staining and in vitro culture of clinical samples, i.e., bone marrow, lymph node aspirate or spleen in visceral leishmaniasis (VL) and dermal and mucous scrapings, aspirates, or biopsy material for cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis. These tools confirm the diagnosis when (i) amastigotes are detected in tissues or (ii) promastigotes are obtained in culture, thus allowing species identification by enzymatic characterization. However, these methods are time-consuming and require culture facilities and individual expertise. Because of these limitations, a number of indirect immunological methods, such as enzyme-linked immunosorbent assay (ELISA), dipsticks and direct agglutination test (DAT), have been developed. Despite the large number of serological tests that are available, there is still no gold standard diagnostic test. This is partially due to the fact that none of these tests is 100% sensitive and specific (Gangneux *et al.*, 2003). Moreover, the spread of *Leishmania*/HIV co-infection complicates the use of the serological techniques as a result of low or lack of antibody responses of these patients (WHO, 2000).

Microscopy and culture have the limitations of low sensitivity and are time consuming. The immunological methods fail to distinguish between past and present infections and are not very reliable in immunocompromised patients. While the molecular approach is capable of detecting nucleic acids unique to the parasite, and would address these limitations. A variety of nucleic acid detection
methods targeting both DNA and RNA have been developed. Amongst these, the PCR has proved to be a highly sensitive and specific technique. The PCR assays can detect parasite DNA or RNA a few weeks ahead of appearance of any clinical signs or symptoms. Different DNA sequences in the genome of leishmania like ITS region, gp63 locus, telomeric sequences, sequence targets in rRNA genes such as 18s rRNA and SSU-rRNA and both conserved and variable regions in kinetoplast DNA (kDNA) minicircles are being used by various workers. (Attar et al, 2001; Santos-Gomes et al, 2000; El Tai et al, 2001; Pizzuto et al, 2001, and Wortman et al, 2001).

1.7.1 Parasitological diagnosis:

1.7.1.1 Microscopy:

The commonly used method for diagnosis of leishmaniasis is the demonstration of parasites in splenic or bone marrow aspirate. Lymph nodes aspirates, liver biopsy or, the buffy coat of peripheral blood (in case of VL), or aspirate specimens (in case of CL & PKDL) stained with Giemsa stain (Sundar and Rai, 2002).

Different diagnostic methods were evaluated in the Sudan and variable sensitivities were observed, lymph node aspirates had a sensitivity of 53-58%, bone marrow aspirates had sensitivity 53-70% as reported in two studies in Sudan (Zijlstra et al, 1992), and spleen aspiration reached a sensitivity of 95-97% (El Hassan et al, 2001). In contrast, smears of skin biopsy material from PKDL patients showed sensitivity of only 30 % (Osman et al, 1997) while examination
of paraffin embedded skin biopsies from PKDL patients revealed *Leishmania* parasites in 20% of tested samples (Ismail *et al*, 1997).

### 1.7.1.2 Culture of leishmania parasites:

Culture of parasite can improve the sensitivity of detection of the parasite; *Leishmania* strains can be maintained as promastigotes in artificial culture medium. Bone marrow, splenic or lymph node aspirates for VL patients and skin biopsies for PKDL and CL patients could be inoculated into culture media for isolation and typing of the parasite. The used culture media include monophasic (Schneider’s insect medium, M199, or Grace’s medium) or diphasic (Novy-McNeal Nicolle medium and Tobies medium). The parasite can also be demonstrated after inoculation of laboratory animals (such as hamsters, mice or guinea pigs) with infected specimen (Sundar and M. Rai, 2002).

### 1.7.2 Leishmanain Skin test:

The Montenegro skin test (leishmanin skin test) tests the delayed hyper sensitivity (DTH) specific to leishmaniasis. In this method, 0.5 ml of phenol-killed whole parasites (5 -10^7 promastigotes) is injected on the volar aspect of the forearm of the patient. After 48 to 72 h, the size of induration is measured and compared with the size of induration produced by injection of a phenol-saline control in the other forearm (Sundar and Rai, 2002).

The test could be positive during CL infection and is useful for follow-up in vaccination programs (da Costa *et al*, 1996). Results of the Montenegro test in acute VL infection are always negative.
1.7.3 Serological diagnosis:

1.7.3.1 Antibody detection:

1.7.3.1.1 Enzyme linked immunosorbant assay (ELISA):

ELISA has been used as a potential serodiagnostic 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. (Whole promastigote and promastigote lysate) The commonly used antigen is a crude soluble antigen (CSA). The sensitivity of ELISA using these concentrations of CSA is reported to range from 80% to 100%, but cross-reactions with sera from patients with trypanosomiasis, tuberculosis, and toxoplasmosis were reported (Bray et al, 1976; Bray et al, 1985).

1.7.3.1.2 rK 39:

The recombinant antigen, rK39, has been shown to be specific in detecting antibodies in patients with VL. It is highly sensitive and predictive of the onset of acute disease. The antigen is derived from L. chagasi (Alborzi et al, 2006).

1.7.3.1.3 Direct agglutination test (DAT):

The direct agglutination test is a simple, fast and economic technique that was described by Allian and Kagan (1975) and modified by ElHarith et al (1986) for the diagnosis of visceral leishmaniasis.

In this test, the trypsinized whole promastigotes are formalin fixed and stained with Coomasie brilliant blue; serum from the patient is then incubated with the antigen, and agglutination is observed the next day. Use of a 0.8% concentration of 0.1 M 2-mercaptoethanol in the sample diluent further improves its
performance. DAT in various studies has shown to be 91% to 100% sensitive and 72% to 100% specific (Badaro, 1996).

Freeze-dried antigens developed in Belgian and Dutch laboratories are likely to overcome some problems like difficult field conditions, the fragility of aqueous antigen, the lack of cold chain, and batch to batch variations in the antigen (Mengistu, 1990).

1.7.3.1.4 Indirect immuno fluorescent antibody test (IFAT):

The IFAT is one of the most sensitive tests available for diagnosis of VL. It is based on detecting antibodies in very early stages of the infection (Williams, 1995). The higher sensitivity of the amastigote-based IFAT resulted in an earlier diagnosis in the absence of clinical signs (Fernandez-Perez et al, 1999). Nigro et al (1996) found that the results of IFAT and DAT had similar specificity but IFAT had a higher sensitivity and a greater diagnostic significance.

1.7.3.2 Antigen detection:

1.7.3.2.1 Latex agglutination:

Latex agglutination test (KATEX) was developed for detecting leishmanial antigen in urine of patients with VL. It has sensitivity between 68% and 100% and a specificity of 100% in preliminary trials (Attar, 2001). The antigen is detected quite early during the infection and the results of animal experiments suggest that the amount of detectable antigen tends to decline rapidly following chemotherapy (Attar, 2001).
1.7.3.2.2 Immunoblotting:

It was found that sera from visceral leishmaniasis patients recognized numerous antigens with molecular weights ranging from 14-110 kDa (Salotra et al, 1999). Studies with four clinical isolates of *L. donovani* showed that the 70 kDa protein was expressed in all examined strains.

1.7.4 Molecular diagnosis:

Due to the limitations inherited in the techniques used for detection of parasites, new approaches for detection of parasites, such as DNA hybridization, have been attempted since the early 1980s. Although these methods had considerable sensitivity (detecting as few as 50 to 100 parasites) (Lanotte, 1981), their potential use in routine diagnosis is hampered by the complex procedure of hybridization. The development of PCR has provided a powerful approach to the application of molecular biology techniques for diagnosis of leishmaniasis. Primers designed to amplify conserved sequences found in minicircles of kDNA of leishmanias of different species were tested in various tissues of relevance (Sundar and Rai, 2002).

1.8 Immunity to leishmaniasis:

Macrophages play a primary role in the host defense and regulation of immune responses upon activation (Unanue and Allen, 1987). The parasites perform a complex host-parasite interaction inside the severe environment of the phagolysosomes and eventually evade this immune defense mechanism (Alexander, 1992).
Although most of the informations on the immunologic mechanisms upon infection and protection from the *Leishmania* parasites were accumulated from the studies in mice. However, the immune response due to VL and the pathogenesis of the disease in human deviates considerably from the murine model.

Resolution of leishmanial infections requires the expansion of specific type I T helper cells (Th I) that secrete or express on their membrane lymphokines capable of activating macrophages that contain these parasites to a microbicidal state. Specific CD8+ T cells, which are triggered during infection, also appear to play a role in protective immunity, possibly through their ability to secrete interferon-gamma. In the mouse model of infection with *Leishmania major*, the expansion of specific type II T helper cells exacerbates disease, an effect that result from the properties of type II T helper derived lymphokines to deactivate macrophages and inhibit release of activating cytokines by type I T helper cells. In the mouse, destruction of intracellular *Leishmania* by activated macrophages depends upon the L-arginine-dependent production of nitric oxide. Molecules from the parasite that can induce, and are the target of, the protective T-cell response have been characterized (Locksley, 1991).

Enhanced induction of IL-10 and IL-4 mRNA in tissues and elevated levels of IL-4, IL-10, and IgE over IFN-γ in serum (Ghalib et al, 1993) suggested that a dominant Th2 response suppresses the activity of Th1 during disease.

With successful drug therapy, T-cell proliferation and IL-2 and IFN-γ production in response to *leishmania* antigen are restored. Cured individuals, however, show
leishmania-reactive T cells with Th1 and Th2-type lymphokines coexisting after infection (Kemp et al, 1993). Thus, the heterogeneous set of cytokine responses provoked by kala-azar during disease and resolution of infection reflects a complex Th1-Th2 cell picture that is difficult to delineate for indicators of clinical improvement.

1.8.1. Humoral Immune response in leishmanaisis:

Infection of Leishmania in human is characterized by the appearance of anti-leishmanial antibodies in the sera of the patients. In CL, usually they are present at low levels during the active phase of the disease (Behin, 1989). However, in some studies the presence of antibodies against L. braziliensis infection in the sera of infected patients has been critically monitored and utilized for the diagnosis and prognosis of the disease (Montoya, 1997; Romero, 2005). Contrastingly, strong anti-leishmanial antibody titres are well documented in VL (Bray, 1976; Neogy, 1987).

Although it is known that the Th1 cytokine interferon-gamma (IFN-γ) probably upregulates isotypes IgG1 and IgG3, and the Th2 cytokines IL-4 and IL-5 stimulates the production of IgG4 in human (Abbas et al, 1996; Rothman et al, 1996). The role of the elevated anti-leishmanial antibodies in kala-azar patients towards protection or pathogenesis is still unclear.

Hailu et al, (2001) attempted to utilize the relative abundances of the anti-leishmanial IgG subclasses to discriminate among the immunity of the active and cured patients as well as the endemic healthy individuals and showed that a low
cell mediated immunity (CMI), in terms of delayed type hypersensitivity (DTH) is correlated with high IgG1, IgG3 and IgG4 and vice versa.

As it is still debatable whether these antibodies have any role in the protection of the disease, a recent experimental study postulated that IgG not only fails to provide protection against this intracellular pathogen, but it actually contributes to disease progression. Passive administration of anti-leishmanial IgG resulted in larger lesions in BALB/c mice with greater amount of IL-10 production (Miles et al., 2005). This result can be correlated with the highly elevated titres of anti-leishmanial antibodies during the active phase of the disease and a consecutive fall in the antibody titre after a successful cure. In addition, studies have shown that large CL lesions were correlated with a higher frequency of lymphocytes producing *leishmania* soluble antigen specific inflammatory cytokines (IFN-γ or TNF-α) (Antonelli et al., 2005).


Anam *et al.*, (1999) investigated the levels of immunoglobulin subclasses in Indian Kala-azar patient sera during disease, drug resistance, and cure. Acute-phase sera showed strong stimulation of IgG, followed by IgE and IgM and lastly by IgA antibodies with expression of all of the IgG subclasses, with a predominance of IgG1 during disease. Furthermore drug resistance was associated with a reduction in IgG2 and IgG3 antibodies, with no significant change in titers of IgG, IgM,
IgA, IgE, and IgG4. A successful cure corresponded with a decline, most significantly, in the levels of IgE, IgG4, and IgG1.

The natural course of humoral immunity in VL could provide valuable data that could be used for improvement of diagnosis, prognosis, and management of VL.

1.8.2 Cell Mediated Immune response in leishmaniasis:

Despite the differences between CL and VL, resistance to disease in both the forms of leishmaniasis is marked by a dominant Th1 response. Severe manifestation of CL is associated with a strong Th2 compared to a predominant Th1 response in the mild manifestation of the disease (Gaafar et al, 1995) On the other hand, it is well documented that VL is characterized by suppression of CMI, which is proved from the unresponsiveness of the patients to the Leishmanin Skin Test (LST) or Montenegro test (Manson-Bahr, 1961).

Containment of the disease following a successful treatment is associated with a strong cell mediated Delayed Type Hypersensitivity (DTH) response (Reiner, 1995).

The two functionally distinct CD4+ T cell subsets, T helper 1 (Th1) and T helper 2 (Th2), are distinguishable by the pattern of cytokines they produce upon stimulation (Mosmann and Coffman, 1989).

Th1 cells are characterized by secretion of IFN-γ and lymphotoxin that are known to activate host defences against intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13 (Cherwinski et al, 1987) that favour the development of humoral responses against extracellular pathogens and also has antagonize the action of IFN-γ and suppress macrophage activation , therefore Th2 may function
as physiologic regulators of immune responses by inhibiting potentially injurious action mediated by Th1 responses (Abbas et al., 1996).

Activation of macrophages to a parasiticidal state by IFN- γ is the main anti-
Leishmania effector mechanism. Indeed, IFN- γ has been demonstrated to increase the synthesis of inducible nitric oxide synthase (iNOS) leading to the L-arginine dependent production of reactive nitrogen radicals toxic for the parasite (Assreuy et al., 1994). IFN- γ which in turn acts on macrophage to induce IL-12 secretion which binds to naïve CD4+ T cells and promotes their differentiation to Th1 cells. The role of IFN- γ in conferring resistance to infection with L. major was demonstrated by showing that genetically resistant mice lacking either the IFN- γ or the IFN- γ receptor gene are unable to control parasite growth (Swihart et al., 1995). The IFN- γ mediated activation of macrophages can be regulated by other cytokines produced by Th2 cells such as IL-4 and IL-10 (Liew et al., 1989).

Th1 and Th2 CD4 T cells develop from common naïve CD4 T cell precursors (Kamogawa et al., 1993). Several parameters have been reported to influence the pathway of differentiation of CD4 T cell precursors, including the type of antigen presenting cells (Moser and Murphy, 2000), the nature of the costimulatory signals (Kuchroo et al., 1995), the extent of T cell receptor (TCR) engagement (Pfeiffer et al., 1995), the dose of antigen (Hosken et al., 1995), the route of antigen administration (Gue´ry et al., 1996), and the number of cell division (Bird et al., 1998). Among these different T cell polarising signals and cytokines have been recognized as crucial inducers of CD4 Th1 and Th2 cell differentiation.
1.10 Treatment of leishmaniasis:

Pentavalent antimony compounds (Sb), were first used for treatment of leishmaniasis in the early 1900s and continued to be the drugs of choice for cutaneous and visceral disease. The most widely used pentavalent antimonial agents used today are sodium stibogluconate (Wellcome Foundation, United Kingdom) and meglumine antimoniate (Rhône Poulenc, France) (Berman et al., 1988). The sodium stibogluconate dose is 20-mg/kg body weight/day, while the duration varies from one endemic area to another, partly reflecting variability in parasite sensitivity. The reported treatments were: 4 weeks in Kenya (Anabwani et al., 1983), 15-30 days in Sudan (Zijlstra et al., 1993; Seaman et al., 1996) and 40 days in India (Thakur et al., 1988). Currently, WHO recommends a dose of 20 mg / kg body weight/day for 4 weeks (WHO, 1990). A longer or shorter course of treatment may be necessary depending on the situation, but one must be aware that longer courses can increase the risk of drug-related toxicity, while shorter courses may risk a relapse or poor response.

1.10 Leishmaniasis control:

Improved control reduces both mortality and morbidity. It also reduces the role of humans as a reservoir in anthroponotic cycles and makes it possible to avert progression of the disease to complicated cutaneous forms. The combination of active case detection and treatment is the key to control. Nevertheless, even that seemingly simple approach faces major obstacles. Although during their initial phases, leishmaniases respond well to treatment, many patients are unaware of the initial symptoms. Furthermore, health systems are frequently either poorly staffed
and lack equipment or are non-existent in remote rural areas where contact with sandflies is most common. (WHO, 2006).

Spraying houses with insecticide is the most widely used intervention for controlling sandflies that are endophilic (rest mostly indoors after feeding). House spraying with the pyrethroid lambdacyhalothrin reduced the risk of cutaneous leishmaniasis, (Reyburn, et al., 2000) sandflies are endophagic (mainly feed indoors) and most active when people are asleep, bed nets provide considerable protection. For example, a case-control study in Nepal showed that people using untreated nets were 70% less likely to develop visceral leishmaniasis than people without nets. (Bern et al, 2000).

In the absence of a reliable and rapid tool for detecting infected dogs, alternative control strategies for zoonotic visceral leishmaniasis are being sought. Dipping dogs in insecticide or applying topical insecticide lotions can substantially reduce sandfly bites on dogs and so protect them from infection, but this strategy requires regular retreatment as the insecticidal effect is short lived (Reithinger et al, 2001).

1.10.1 Vaccine:

Of all the parasitic diseases, leishmaniasis is considered the most likely to succumb to vaccination. The parasite has a particularly simple life cycle, resolution of primary cutaneous leishmaniasis usually results in resistance to re-infection, and studies in experimental models have suggested simple CD4 Th1-type, cell mediated resistance (involving activation of macrophage killing mechanisms by T lymphocyte-derived interferon ?). In experimental models of cutaneous leishmaniasis, in which CD4 Th1 responses are driven towards a
polarised Th1 response, protection can indeed be achieved by vaccination, although this rarely results in complete protection from development of lesions. Such vaccines, however, stimulate only poor memory, and protection wanes after a few weeks. (Handman, 2001) In primate studies and clinical trials they show immunogenicity but rarely give appreciable protection. (Kenney et al, 1999), (Belkaid et al, 2002).

**Rationale:**

Visceral leishmaniasis patients present a strong humoral response to *Leishmania* infection. The induced anti *leishmania* antibodies directly correlates with the severity of the disease and inversely related to cure and protection. The current diagnosis methods of VL are based on detection of anti-*Leishmania* antibodies in serum of suspected patients and demonstration of the parasite in lymph nodes, spleen or bone marrow aspirates. Patients usually reports for diagnosis after the development of the clinical symptoms and no data is available about the time of infection or incubation period. Furthermore, the course of the induced antibodies during treatment and after cure is not known. Monitoring of the antibody evolution of VL could provide valuable data that can improves the diagnosis, predict the prognosis and improve the management of VL patients.

**General objectives:**

To improve the diagnosis and management of Visceral Leishmaniasis through monitoring the evolution of the humoral immune response of healthy individuals living in visceral leishmaniasis endemic area in Dinder National Park in central Sudan.
Specific objectives:

1- To monitor the seroconversion of individuals living in VL endemic area.

2- To monitor the course of sero reactivity and development of clinical disease.

3- To detect the *leishmania* parasite DNA in blood of individuals living in VL endemic area.
Chapter Two

MATERIALS AND METHODS

2.1 Study area and population:
This study was conducted in Dindir National Park in central Sudan. The park is a wild animal reserve and known to be hyperendemic of visceral leishmaniasis. The Park is known to be highly endemic of zoonotic Visceral Leishmaniasis, due to the large number of animals that can act as reservoir hosts for the diseases, while sandflies are the vectors of parasites. The study was approved by the research ethics committee of the Institute of Endemic Diseases, University of Khartoum. All participants were recruited according to their residence in the park.

One hundred and ten (110) wild animal guards working in Dindier National Park were consented and recruited to the study. Most of them are working inside the Park. Every participant was screened for exposure to Leishmania infection by clinical examination, detection of anti Leishmania antibodies using the DAT test and ELISA. The participants were followed for 12 months by visits the campus. In each visit the participants were physically examined and screened for Leishmania antibodies using DAT. The induced antibody titers and immunoglobulin classes and subclasses were measured using ELISA. PCR was used to detect Leishmania parasite kDNA in the samples.

2.2 Study design:
A longitudinal prospective study was conducted for 12 months.
2.3. Sampling method:

Samples were taken from wild animal guards, male and females (figure 2) aged 20-60 years throughout the two surveys, which were carried out in June 2009 and June 2010.

1. First trip was started with a sample size of 51; female [5], male [46].
2. Second trip sample size of 59; female [4], male [55].
3. There were thirty-one samples collected twice in both surveys.

2.4 Study subjects:

One hundred and eleven (110) new appointed and continue working guards were consented and recruited to the study in the two surveys.

2.5 Recruitment of participants:

The participants were recruited according to their residence in the park.

2.6 Ethical clearance:

This study was approved by the research committee of the Institute of Endemic Diseases, University of Khartoum. Informed verbal consent was obtained from the wild animal guards department.

2.7 Inclusion criteria:

1. Adults, age 20 to 60 years.
2. Healthy with no serious health problem.

2.8 Methods of data collection and sample:

Data was collected via:

1. Questionnaire filled by personal interviews.
2. Blood was collected on Whatman No.3 filter papers.
2.9 Diagnosis of VL:

2.9.1 Physical examination:

Medical examination of the participants was done by qualified medical doctor. The body temperature, the size of lymph nodes, spleen, and liver were recorded.

2.9.2 Elution of blood sample from filter paper:

Circles of 1cm diameter were bunched from Whatman No.3 by paper punch. Filter papers soaked in donor blood (equivalent to 5 µl blood). The punched papers were inserted in 1.5 ml eppendorf tubes and incubated with 125 µl normal saline for 18 hours. The eluted sera (from the filter paper) were serially diluted into the V-shape wells of microtitre plate.

2.9.3 Direct agglutination test (DAT):

DAT was carried out as described by El Harith et al, (1986) in V-shaped 96 well microtiter plates. Serum samples were serially diluted (1:100 to 1:6553600) in 0.2% gelatin containing 0.9% NaCl (w/v) and 0.78% (v/v) 2-mercaptoethanol. 1= 3200 serum dilutions used as the cut off positive sample.

2.9.3.1 Performance of the DAT:

Fifty µl of serum diluents (0.9% NaCl, 0.2 % Gelatin and 0.8% 2ME) were dispensed into the V-shape wells of microtitre plate; rows 1 and 3-12 (A-H). 75 µl of the diluent was dispensed into row 2 (A-H), and then 25µl of eluted sera (test and control samples) was added in row 2(A-H). After mixing with multi channel micro pipette, 50µl was transferred to row 3. This was repeated until row 12; from which 50 µl were discarded. 50µl of the antigen were added in all wells. The plate was gently tapped on all sides in order to mix the content and incubated for 18
hours at room temperature. Serial dilution of the samples starting from 1:100 to 1:102400 were achieved. The agglutination activity was determined visually and the titer of the reaction was recorded for each sample.

2.9.3.2 Interpretation of DAT results:

A cut off titer =1:3200 was considered as a positive result

2.9.4 ELISA for parasite-specific Igs:

Enzyme-linked Immunosorbant assay (ELISA) was done to measure IgG, IgM, and IgG subclass antibodies to *Leishmania* in collected serum samples. The test was carried out on polystyrene round-bottom microtiter plates (Tarsons). Antigen extracted from *L. donovani* was applied to the plates at 20 mg/ml in 0.02 M phosphate buffer (PH 7.5) and incubated at 4°C overnight. Following 3x washes the plates with PBS supplemented with 0.05% Tween 20, excess reactive sites were blocked with 2% gelatin for 1h at room temperature, washed as before, and subsequently incubated overnight at 2h at room temperature with kala-azar sera serially diluted in PBS containing 1% gelatin. After washing, peroxidase-conjugated goat polyclonal antibodies directed against human IgG and IgM (Sigma immunochemical, St. Louis,Mo.) were applied at a 1: 5,000 dilution in PBS for 3 h at room temperature.

After four washes, o-phenylenediamine dihydrochloride were added as an enzyme substrate for 45 min, and the optical density was read at 450 nm in ELISA reader (Thermal Labsystem- Multi Ascent model NO 354).

For the determination of human IgG subclass antibodies, the washed wells were incubated with serially diluted. Kala-azar sera were probed with mouse anti
human IgG subclass-restricted monoclonal antibodies (sigma immunochemical) at a 1:3,000.

Dilution for 3 h at room temperature. After three washes, peroxidase-conjugated goat anti-human IgG (sigma immunochemicals) was applied at 1:5,000 dilution overnight at 4°C. The color reaction was carried out as described above, and the optical density was read at 450 nm. Titers were determined from the extensive titration of each serum sample as dilution of serum required to reach half-maximal absorbance.

2.9.5 DNA extraction:

Two methods of DNA extraction were used to detect subclinical infection.

2.9.5.1 Phenol chloroform method:

Using sterile puncture, 2 pieces from the blood drops of filter paper were placed in sterile labeled eppendorf tube and then 245 ul lysis buffer was added. 2.5 ul of Triton X-100 were added. 2.5 ul of proteinase K then were added and incubated at 60°C overnight.

In the next day an equal volume (250 ul) of buffered phenol were added and shaked gently for at least 2 minutes and centrifuged at maximum speed for 2 minutes, the aqueous phase were transferred to a clean labeled tube and the organic phase were discarded. An equal volume of chlorophorm - phenol mixture (phenol: chlorophorm, 1:1 v / v) were added and centrifuged as in the last step. Then an equal volume of chloroform were added and centrifuged as before.

The DNA were precipitated by adding double volume of cold absolute ethanol and mixed gently and incubated overnight at –20°C.
On the third day the samples were thawed and centrifuged at maximum speed 4000 rpm for 20 minutes. The supernatant were discarded and the tubes were filled half away with 70 % ethanol and centrifuged at 12000 rpm for 15 minutes, this step was done twice. The tubes were kept on the bench at room temperature until the last traces of fluid evaporated .Then the DNA pallet (which is often invisible) were dissolved in DW. The DNA was then stored at -20 °C until used.

2.9.5.2 Chleix method:

100 ul of a 5 % (w/v) Chelex-100 solution (sigma) were added to 1.5 ml microcentrifuge tube and placed in heating block at 100°C for 5 minutes. A circle of 1cm diameter was bunched from whatman No.3 filter papers soaked in donor blood (equivalent to 5 µl blood). The filter papers were then immersed in the hot chelix solution. The tube was capped, gently shaken for 30 seconds, and then returned to the heat block for 10 minutes. The samples were centrifuged at 12.000 rpm for 2 minutes and the supernatant was removed to a new microcentrifuge tube and spun again at 12.000 rpm for 2 minutes. The supernatant was removed to a new microcentrifuge tube and either used immediately in amplification reaction or stored at -20°C until used.

2.9.6 PCR:

kDNA PCR was done as described by Smyth et al (1992) using genus specific primers for mini-circle kinetoplast (kDNA) the AJS3

5'ggggTTggTgTAAAATAgggC-3' and DBY 5'CCAGTTTCCCgCCCCggAg-3' primers. The reaction volume was 50 ul per sample in 0.5 ml thin walled micro
centrifuge tube. The mixture consisted of 5ul of 10x reaction buffer, 2ul of 20mM dNTP mix (ABgene-0196-UK), 3ul of 25mM MgCl$_2$ to a final concentration of 1.5ul. 2.0ul of primer mixture (10mM each), to a final concentration of 0.5uM each and 0.25ul of thermo-stable DNA polymerase (5U/ul). For each PCR tube 3ul of template DNA were added; PCR mixture was then completed to 50ul with double distilled water. PCR was done using Perkin Elmer thermal cycler (480-USA). The amplification was done for 35 cycles. The DNA was initially denatured at 94$^\circ$C for 3min. Each cycle included annealing at 67$^\circ$C for 1min, extension at 72$^\circ$C for 1min and denaturation at 94$^\circ$C for 30sec. A final extension cycle at 72$^\circ$C for 10 min was run. The PCR products were stored at 4$^\circ$C until analyzed.

2.9.7 Gel electrophoresis:

90 ml distilled water and 10 ml 10X Tris boric EDTA (TBE) were added to 1.0 gram of agarose. The mixtures were melted and 3ul of Ethidium Bromide (10mg/ml) were added to the gel and mixed, the mixture was then poured into horizontal electrophoresis tank and the gel was left to polymerize. 7µl of PCR product were mixed with loading buffer and loaded into the gel. 10µl of DNA molecular weight marker was also loaded. Running buffer was added containing 95ml distilled water and 5 ml 10x TBE buffer. The run were performed at 80 Volt and the current range was from 3-8 mA for 30 minutes. The gel was then visualized over ultra violet transilluminator (UVP) and photographed.
2.10 Data analysis:

The data were entered, verified and analyzed using SPSS version 18 and Sigma Plot version 11.
Chapter Three

RESULTS

3.1. Study Participants:

Two surveys were conducted in June 2009 and June 2010.

Fifty one wild animal guards with previous VL history or not (figure 3) were recruited during the first trip and fifty nine were recruited during the second survey. Thirty one participants were sampled during the two surveys. The age range of the participants ranged between 20 – 60 years.

3.2.1 Direct agglutination test (DAT) results:

In the first survey the number of positive reactors (DAT titer = 1:3200) were 22(43.1%) out of 51.

While the number of positive reactors in the second survey were 24(26.6%) participants out of 90. Twenty guards were missed in the second survey and only 31 were sampled twice. Fifty nine (59) new guards were sampled in the second survey.

Of the thirty-one (31) cohort sampled in two surveys, the number of positive DAT reactors in the first survey were 12 ;( 38.7%) participants, 4 (19.35%) converted to negative during the second survey.

3.3. Enzyme-linked immunosorbent assay (ELISA) results:

3.3.1 Enzyme-linked immunosorbent assay (ELISA) results for IgG:

In the first survey 28 ;( 54.9%) participants were positive in IgG ELISA, while 57 (63.3%) were positive in the second survey.
3.3.1.1 Conversion of ELISA for IgG result:
Of the thirty-one (31) cohort, 19 ; (61.3%) participants were positive IgG ELISA in the first survey. 6 ; (19.35%) were reactive in the first survey and converted to negative in the second survey. Nine 9 ; (29%) were non reactive in first survey and converted to positive in second survey. While 11 (35.5%) were reactive positively in IgG ELISA in both surveys.

3.3.2 Enzyme-linked immunosorbent assay (ELISA) results of IgM:
All sample tested negative for anti-leishmania IgM in the first survey, 2 (2.2%) were reactive in the second survey.

3.3.3 Enzyme-linked immunosorbent assay (ELISA) results of IgG:
Samples were had no anti-leishmania IgG during the first survey, while in the second survey 16 (17.8%) samples were reactive.

3.3.3.1 Conversion of ELISA for IgG results:
Of the 31 participants were negative in IgG ELISA in the first survey. In the second survey 7 ; (22.6%) were reactive positively in IgG ELISA.

3.3.4 Enzyme-linked immunosorbent assay (ELISA) results for IgG2:
ELISA for IgG2 was not done for the sample collected during the first survey. While in the second survey 38 (42.2%) participants were reactive positively in IgG2 ELISA.

3.3.4.1 Conversion of ELISA for IgG2 result:
Of the 31 participants in the second trip 14 (45.2%) were reactive for antileishmania IgG2.
3.3.5 Enzyme-linked immunosorbent assay (ELISA) results for IgG3:
Samples were negative for IgG3 in the first survey, and 12 (13.3%) were reactive for IgG3.

3.3.5.1 Conversion of ELISA for IgG3 result:
None of the 31 cohort members was reactive for IgG3 during the first survey. 3 (9.7%) were reactive positive for IgG3 in the second survey.

3.3.6 Enzyme-linked immunosorbent assay (ELISA) results for IgG4:
None of the samples were IgG4 reactive during the first survey, 3 (3.3%) converted to positive during the second survey.

3.3.6.1 Conversion of ELISA for IgG4 result:
All members of the cohort were none reactive in the first survey, while 2 (6.5%) were converted to positive in the second survey.

3.4. Polymerase chain reaction (PCR) result:
None of the samples had detectable parasite DNA.

Figure 2. Gender distribution of the participates.
Figure 3. Previous visceral leishmaniasis cases among participants.
Figure 4. Detection of anti-\textit{Leishmania} antibodies in sera of the participants of the first survey using ELISA.
Figure 5. Detection of anti-Leishmania antibodies in sera of the participants of the second survey using ELISA.
Figure 6. Detection of anti-Leishmania IgG subclasses in some of participants of the second survey using ELISA.
Table 1. Anti-<i>Leishmania</i> antibody titre as detected by direct agglutination test (DAT) of 31 individuals in the first trip and the same 31 individuals in the second trip.

<table>
<thead>
<tr>
<th>Trip</th>
<th>1/1600 or less</th>
<th>1/3200</th>
<th>1/6400</th>
<th>1/12800</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st survey</td>
<td>NO</td>
<td>%</td>
<td>NO</td>
<td>%</td>
</tr>
<tr>
<td>19</td>
<td>61.3%</td>
<td>5</td>
<td>16.1%</td>
<td>5</td>
</tr>
<tr>
<td>2nd survey</td>
<td>23</td>
<td>74.2%</td>
<td>6</td>
<td>19.4%</td>
</tr>
</tbody>
</table>

Table 2. Results of 31 participants in first and second survey for IgG total, IgM, IgG1, IgG2, IgG3 and IgG4 using ELISA.

<table>
<thead>
<tr>
<th>survey</th>
<th>ELISA</th>
<th>IgG</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NO %</td>
<td>NO %</td>
<td>NO %</td>
<td>NO %</td>
<td>NO %</td>
<td>NO %</td>
</tr>
<tr>
<td>1st survey</td>
<td>+ve</td>
<td>19 61.3%</td>
<td>0 0%</td>
<td>0 0%</td>
<td>N.D</td>
<td>N.D</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>12 38.7%</td>
<td>31 100%</td>
<td>31 100%</td>
<td>N.D</td>
<td>N.D</td>
<td>31</td>
</tr>
<tr>
<td>2nd survey</td>
<td>+ve</td>
<td>20 64.5%</td>
<td>1 3.2%</td>
<td>7 22.6%</td>
<td>14</td>
<td>45.2%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>11 35.5%</td>
<td>30 96.8%</td>
<td>24 77.4%</td>
<td>17</td>
<td>54.8%</td>
<td>28</td>
</tr>
</tbody>
</table>
The outcome of infection by *Leishmania* parasites depends on the infecting parasite species and the host immune response to the infection. The majority of individuals infected with *Leishmania* remain asymptomatic. Little data is available on the natural course of infection. This study aimed to determine the rate of asymptomatic infection among individual living in endemic area. The study was conducted in Dindir National Park. Two cross sectional survey were conducted in June 2009 and June 2010. The park guards were recruited for monitoring their antibody responses to *leishmania* as indicator of exposure to infection, serum samples were collected for measuring antibodies and for detection of *leishmania* DNA using PCR. The rate of DAT seroreactivity of the participants was 38.7% in the first survey and 25.8% in the second survey indicating active transmission of leishmaniasis during the study period .in another study conducted in village at Attbara River reported by El-Safi *et al* (2002) the positive DAT rate was 5%. A similar study conducted in India by Gidwani *et al* (2009) a DAT rate ranged between 10%-26%. The differences in DAT rates could be due to the variation in transmission of infection. The site were this study was conducted is known to be with in high transmission area. An interesting finding of this study was the negative seroconversion of those who tested positive during the first survey. Similar findings were reported by Ahmed (2008). The negative seroconversion could be due to parasite clearance by those individuals mostly
mediated by innate immune responses. Analysis of the innate immunity of those individuals might explain the mechanism of negative seroconversion. In this study about 25.8% of positive DAT participants remained positive through the second survey (with variation in the titer of some of them), those participants seemed to be continuously exposed to the parasite and remain subclinical or asymptomatic without developing clinical VL during the follow up. Similar finding was reported by Zijlstra et al, (1998), who found only 6 out of 12 subclinical subjects remained positive after 12 months follow up.

Furthermore similar were reported by Silva et al (2011) in Brazil who found that 46% subjects from endemic area had serological test positive result they had clinical reassessment 3–4 years after the first evaluation and revealed none of the subjects who tested positive developed clinical VL .The significant titer positive rate of anti-leishmania IgG compared to the lower rate of IgM indicates prolonged exposure to *leishmania* parasites. A similar high rate of IgG was previously reported by Ibrahim et al (1999).

In this study prevalence of the serconverted participants ranged from 19.35%-29%, similar findings were reported by Silva et al (2011) in study conducted in Brazil using ELISArk26. The increase in anti-leishmania IgG subclass detected in the second survey could be used as prognosis marker based on the finding of Hailu et al (2001). Who followed individuals with positive anti-leishmania IgG and later developed clinical VL. The increase in anti-leishmania IgG2 antibody has been shown to be associated with asymptomatic infection (Almeda et al, 2005). Interestingly there was an
increase in both IgG3 and IgG4 indicating mixed Th1 and Th2 response of exposed individuals.

This study failed to detect circulating parasite DNA using PCR. This finding was expected since the participants were apparently healthy and the parasite load was either low or the parasite might have been cleared. The detected antibodies classes and subclasses reflect exposure of the participants and the follow-up was needed to detecting the long term impact on protection and susceptibility to clinical VL.

This study had limitation regarding the sample size as well as loss of some participants during the follow-up. The period of follow-up needed to be extended to determine the outcome of the infection.
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