

ISSN 1682-296X (Print)  
ISSN 1682-2978 (Online)



# Bio Technology



**ANSI***net*

Asian Network for Scientific Information  
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

## Evaluation of Gene Targeted PCR and Molecular Hybridization Used in Diagnosis of Human Leishmania Isolates

N.M. Abdalla

Department of Microbiology and Medical Parasitology, College of Medicine,  
King Khalid University, Abha, Saudi Arabia

**Abstract:** This study aimed to identify cases of leishmaniasis in the Nuba Mountain area, which is situated in a unique geographical site as it is located in the centre of Sudanese leishmania belt. This study applied in field situation some of highly powerful molecular tools to diagnose cases and typing of species. The study was conducted in the Green Valley village (Rashad Province, South Kordofan State) with a population of 332. Most of the villagers have presented with sub-clinical form of leishmaniasis, presented with minor symptoms and signs that can occur in clinical form of visceral leishmaniasis such as fever, diarrhoea, epistaxis, enlarged lymph nodes, spleen and liver. As many conventional diagnostic methods such as direct microscopy, culture and serology have some drawbacks and failures in diagnosing subclinical cases of leishmaniasis, here in this study molecular techniques such as PCR and hybridization have been utilized and assessed on blood spotted finger pricks spotted on filter papers collected from the villagers under field condition as well aspirates from lymph nodes were fixed, stained with Giemsa and examined microscopically to visualize LDBs (Leishmania Donovanii Bodies). Low parasitemia and poor sampling technique have led to the presence of invalid samples that have been discarded by checking initially the presence of enough human DNA on these samples by using human DNA specific primers (AME2 and AME206). Then, valid samples were tested for the presence of leishmanai parasite (donovani complex) specific primers (AJS3 and DB8), further more the *L. infantum* specific probe (EH) and *L. donovani* complex specific probe B4 Ras were applied to these samples blotted on nitrocellulose membrane, the former yielded negative outcome on X-ray films which means that *L. infantum* is not present among the studied samples while, the later have recognized a lot of positives although, these samples were negative by ordinary gel visualization. Only one positive case detected microscopically and the final positive cases detected by PCR and hybridization tests were 32 out of 332 belong to donovani species. No *L. infantum* detected. Almost, all of the 32 positives have presented with sub-clinical pattern of the disease, indicating the predominance of sub-clinical form in this study area.

**Key words:** Leishmaniasis, PCR, hybridization, probe, subclinical, Nuba mountain

### INTRODUCTION

Leishmaniasis is a parasitic disease transmitted by female sandfly of the genus *Phlebotomus* in old world and *Lutzomyia* in new world. The disease is worldwide distributed and endemic in 82 countries. The annual incidence is estimated at some 600,000 new clinical cases, officially reported of 12 million cases and a population at risk about 350 million (WHO, 1990). The disease has a major public health problem special in remote areas leading to major socio-economic and health impact in communities found in developing countries.

In Sudan, leishmaniasis was studied long time ago (Neave, 1904; Kirk and Sati, 1940). The visceral leishmaniasis hyper-endemic foci are mainly in East and South of the country (El-Hassan and Zijlstra, 2001; Seaman *et al.*, 1996). A longitudinal study was conducted

in Eastern Sudan (Gadarif region) where, high morbidity and mortality were observed from visceral leishmaniasis and presence of large number of post-kalazar dermal leishmaniasis (Zijlstra *et al.*, 1994). An epidemic of visceral leishmaniasis has been ravaging an area of Western Upper Nile province, Southern Sudan (Siddig *et al.*, 1990). In Northern Sudan the disease is mainly occurred in sporadic cutaneous leishmania form (El-Safi *et al.*, 1991). Western Sudan received less attention and studies (Abdalla *et al.*, 2002).

The disease showed wild diversity in clinical pattern, but the pathogenicity depends mainly on parasite genotype versus host immunogenetic profile. Newly utilized molecular techniques are used in many studies which enrolled using gene targeted PCR, probing and hybridization (Barker, 1987). The diagnose visceral leishmaniasis from peripheral blood using the Polymerase

Chain Reactions (PCR) technique was developed for which the detection limit is one leishmania infected macrophage in 8 mL of blood. The sensitivity was determined to be 96% and specificity of 100%. In clinically cured patients who received treatment, 92% of them will show negative PCR within 1-6 months after treatment, this might help to assess the successfulness of the treatment (Passos *et al.*, 1986).

A wide range of investigations are available but still a reliable precise test is needed to play a role in the molecular epidemiology and pathogenicity of this disease (Abdalla *et al.*, 2001, 2002). This study aimed to assess the value and identify some of the newly utilized molecular techniques on a field collected clinical samples from human (Mohammad *et al.*, 2009).

This work was supported by the TDR/WHO grant (Ref.M8/181/4/A.295). The ethical clearance was obtained from The Sudanese National Health Laboratory (Khartoum/Sudan).

## MATERIALS AND METHODS

**The work site:** A small village lies in Nuba Mountain, West of Sudan (12N-9S and 33E-29W). The total population was around 332. The area was selected due to its unique geographical site being in the middle of Sudan leishmania belt.

**Sampling:** Numbering of houses in the village, ID number for all villagers.

Demographic and epidemiological data were collected using special questionnaire (a copy of the questionnaire is attached).

Clinical examination was conducted to all villagers including; body temperature, systemic examination, assessment of enlargement of liver, spleen and lymph nodes. The clinical examination have been conducted by the correspondence author (as he is a medical professional).

**Parasitological test:** Smears made from aspirated materials from lymph node. Lymph node aspirations were done using a syringe fitted with a 21 gauge needle. Air-dried, fixed with ethanol. Smears were prepared from the aspirates. Stained with Giemsa and examined by direct microscopy using oil immersion to detect the LDBs (Leishmania Donovanii Bodies).

**Molecular biology:** A finger prick blood spotted filter papers were collected from all the villagers. All the samples were tested for leishmania parasite detection using specific donovani primers AJS3 (5'CCAGTTTTTCCC GCCCCT3') and DB8

(5'GGGTTGGTG TAAAATAGGGC3')(Barker, Cambridge). Donovani complex probe B4 Ras (85 bp) and Infantum specific probe EH {version: S49390.1 G1: 28802 Pubmed 1331940} were used for parasite identification and typing.

**DNA extraction:** DNA was extracted from blood spotted filter papers collected from all the villagers. SDS detergent and proteinase K were used, after spinning the supernatants were treated with phenol/chloroform/isoamyl alcohol. DNA were precipitated in absolute ethanol and preserved in Tris-EDTA (TE). The chelex extraction method was proved not to be more sensitive when compared with phenol/chloroform/isoamyl alcohol.

**Human DNA detection:** All the field samples were assessed for validity prior to test for the leishmania parasite DNA. Samples DNA extraction was done by the phenol method. The DNA were amplified by PCR using amelogen primers AME2 (740 bp X- chromosome product) and AME206 (550 bp Y- chromosome product) to detect the human DNA in each sample hence, the validity of the sample for Leishmania DNA detection were selected. Total of 24 mL PCR reaction mixture using human DNA primers (AME2 and AME206) composed of: 4 mL dNTPs nucleotides, 2.5 mL 10X Reaction Buffer, 0.25 mL Primer AME206 dilution, 0.25 mL Primer AME2 dilution, 16.8 mL H<sub>2</sub>O, 0.4 mL Taq- polymerase concentration.

PCR reaction conditions were; no hot start. Denaturation temperature 94°C for 1 min, annealing temperature 60°C for 1 min, Extension temperature 72°C for 2 min repeated for 35 cycles. Incubation temperature 72°C for 10 min. Store temperature 12°C for overnight. All samples-DNA were run in 1.4% agarose gels at 80 V and Ethidium bromide was used for detection.

**Leishmania parasite DNA detection by PCR:** To identify the leishmania positive samples, all valid field samples were tested for detection of Leishmania parasite. The selected valid DNA samples were amplified by PCR using kDNA primers (AJS3 and DB8) and Taq polymerase enzyme was added after the first PCR cycle hot start (95°C for 5 min). After the PCR cycles ended the amplified DNA was examined using horizontal agarose electrophoresis technique visualized by Ethidium bromide. DNA profile was compared with known L.d. isolates (positive control). PCR reaction conditions using Leishmania primers (AJS3 and DB8) composed of: Hot start temperature 94°C for 5 min. Denaturation temperature 94°C for 1 min, annealing temperature 64°C for 1 min., Extension temperature 72°C for 2 min repeated for 35 cycles. Incubation temperature was 72°C for 10 min, store temperature 12°C for overnight.

**Contents of 24 mL total PCR reaction mixture:**

Four milliliter d NTPS Nucleotides, 2.5 mL 10XReaction Buffer, 1.5 mL Primer AJS3 dilution, 1.5 mL Primer DB8 dilution, 9.5 mL H<sub>2</sub>O, 5 mL Taq-polymerase dilution. All samples were run in 1.4% agarose gel stained with Ethidium bromide at 80 V.

**Blotting and hybridization:** Special leishmania species probe was used to identify donovani complex. In brief, the PCR products on the agarose gel was blotted on nitrocellulose membrane applied on the gel and a moderate weighs on top of the membrane, this manouver took place on a tray soaked in 10x SSc. With no air trap (minum of 4-6 h) after that the membrane air dried and fixed by UV light (12 J). In epindorff tube with screw cover 5 µL EH- infantum specific probe or 5 µL of B4 Ras donovani complex specific probe (85 bp) was applied to the membrane in hybridization cylinder in which 1 µL klenow enzyme, 10 µL random primer (6 bp), 10 µL reaction buffer and 3 µL radioactive dNTPs plus 21 µL dH<sub>2</sub>O were mixed. The labelling reaction final volume was 50 µL kept in 37°C hot block for 10 min. A hybridization column was used for purification then, a hyper stringency solution composed of 0.1 x SSC plus 0.1 % SDS (sodium dodecyl sulphate) was used for washing the membrane(s) three times each for 15 min then add X-ray film with the film cassette and kept over night or more in -70°C then, the films showed black traces and bands in positive samples.

**RESULTS**

The total number of villagers under study (332) around 48% of them were children (<16 years old). Females were predominant (Table 1). Clinically, both groups (children and adults) did not complain of; weight loss, epistaxis and cough. Only 3% complained of diarrhea. Fever was a complain of 34% of children and 16% adults, while abdominal pain of 6.7% of children and 13.5% of adults. Clinical assessment revealed no cutaneous ulcers, no mucocutaneous lesions and no suspicious skin scars. Systemic examination which includes assessment of liver and spleen enlargement among the study group; 14 individuals sowed enlarged liver while 50 ones showed enlarged spleen (Table 2). Only 63 individuals (51 children and 12 adults) showed enlarged lymph nodes, localized to inguinal region. Almost, all of the studied population have presented with sub-clinical pattern of the disease, indicating the predominance of sub-clinical form in this study area rather than the classical clinical form of leishmaniasis. Detection of LDBs microscopically was found in one villager (a child 5 years old, febrile 38°C, loss of weight, spleen 8 cm, liver

Table 1: Distribution of the study population by gender and age group

Age group	Male No.	Females No.	Total No.
0-5 years	26 (46.4)	30 (53.6)	56 (16.9)
6-10 years	22 (37.9)	36 (62.1)	58 (17.5)
11-20 years	27 (35.1)	50 (64.9)	77 (23.1)
>20 years	71 (50.4)	70 (49.6)	141 (42.5)
Total	146 (43.9)	186 (56.1)	332 (100)

Values in brackets indicate percentage

Table 2: Clinical assessment of liver and spleen among the study group

Organ	Size (cm)							
	0	1	2	3	4	6	8	12
Spleen	282	4	11	1	21	8	2	3
Liver	318	0	7	0	6	1	0	0

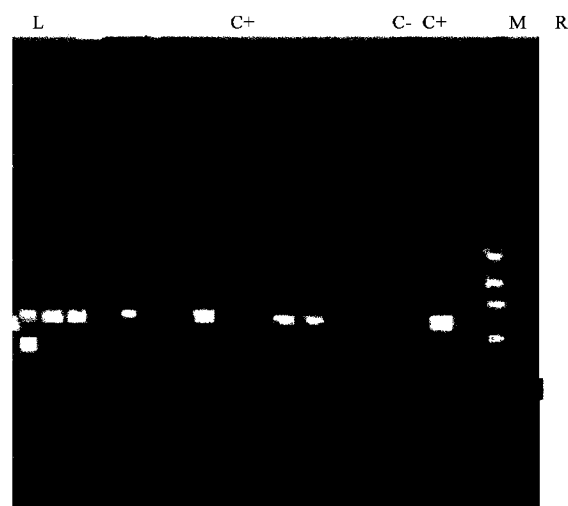


Fig. 1: Human DNA specific primers (AME2 and AME206) double bands: female, single band: male. From left (L) to right (R) 113, 172, 132, 131, 123, 37

4 cm, enlarged inguinal lymph nodes and positive PCR). This child was admitted to Kala-azar ward (Soba Teaching Hospital, Khartoum, Sudan) received treatment and improved. Fever subsided, spleen regressed and gain weight.

The results of the validity assessment of samples using the human DNA primers (AME2 and 206) to detect the sex-chromosome showed; female sex as a single band with heavy molecular weight (bp) while, male sex showed double bands with lighter molecular weight (Fig. 1).

Detection of leishmania parasite DNA in some field samples using kinetoplast DNA primers AJS3 and DB8 (Fig. 2). The hybridization of radioactive B4 Ras probe (donovani complex specific probe) findings on some of the field samples are shown in Fig. 3. The final positive cases detected by PCR and hybridization tests were 32 out of 332 belong to donovani species. No *L. infantum* detected.

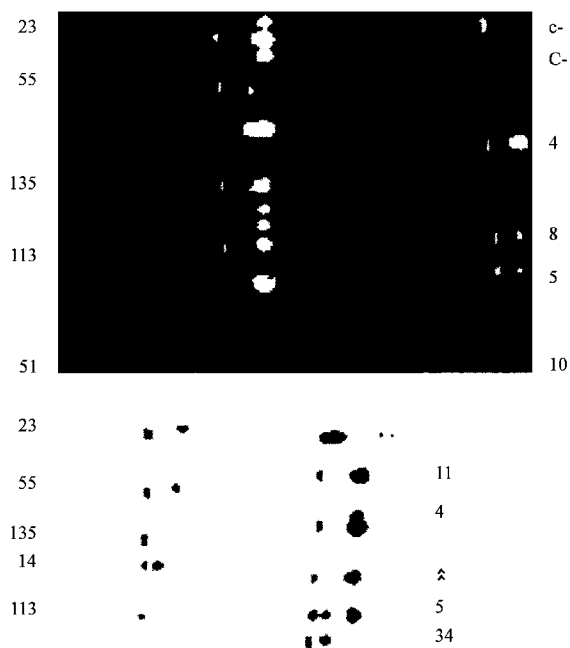


Fig. 2: Upper photo: AJS and DB8 primers applied on field samples to detect leishmania DNA lower photo: 24 h hybridization

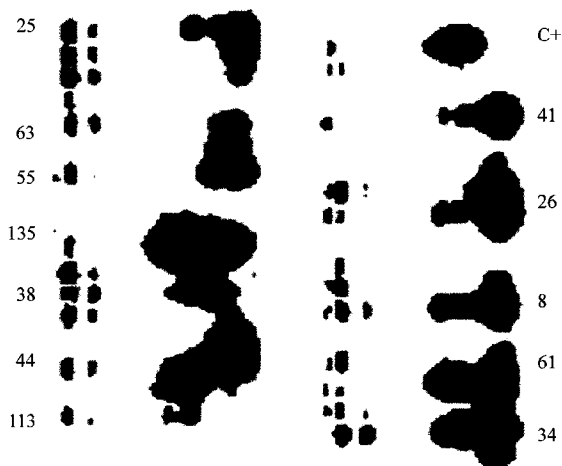


Fig. 3: Five days hybridization on similar field samples, showing positive samples detected by dark band correspondence to the radioactive B4 Ras probe. Positive C+ and negative controls C- are seen on top right of the gel

### DISCUSSION

The clinical pattern of the disease among the villagers understudy (n = 332) showed the predominance of sub-clinical pattern. The disease clinical manifestation is

governed by the host immune reaction. There are T cells subsets (T helpers): TH1 associated with IL2, gamma IFN and mediate Delayed T-cells Hypersensitivity (DTH) immunity to the disease. TH2 associated with IL4, IL5 and promote IgG but do not mediate DTH and most likely a florid clinical pattern of the disease. The Human HLA-pattern (locus on chromosome 6) is a major determinant of individual immune-genetic reaction in diseases and tissue typing. In the other, hand the parasite genotype is also an important determinant of the microorganism virulence and disease presentation. The molecular analysis of leishmaniasis is proved to be highly sensitive and specific on clinical and sub-clinical human samples (Tashakori *et al.*, 2006). The traditional investigation methods used to identify leishmaniasis such as direct microscopy looking for LDBs (leishmania donovani bodies), culture and serology (ELISA, DAT and K39) have their weakness in identifying the positive cases specially if used for subclinical cases where the number of parasites will be expected to be low (Weigle *et al.*, 2002), as well as the problems of culture contamination and cross reaction of serological tests (El-Harith *et al.*, 1987). The DNA extraction and amplification using specific primers (Kinetoplast DNA) is a good screening tool that can be used in variable epidemiological studies further more the species-specific causative agent can be identified and further taxonomic characteristics can be obtained. In this study, the conventional visualization of PCR products on agarose gel using UV light and camera proved to omit some positive samples due to their faint bands. Hybridization technique by blotting the gel on nitrocellulose membrane and using X-ray films to show the radioactive bands have yielded more positive samples (Schoone *et al.*, 1991). In fact, the period of blotting the nitrocellulose membrane with the X-ray film showed more positive bands as long as this period increased. So, five days hybridization with X-ray film is more sensitive in clarifying positive samples in comparison with 24 h period. The *L. infantum* specific probe (EH) and *L. donovani* complex specific probe B4 Ras (Paindavoinne *et al.*, 1986) were applied to the blotted nitrocellulose membrane, the former yielded negative outcome on X-ray films which means that *L. infantum* is not present among the studied samples while the later have recognized a lot of positives although these samples were negative by ordinary gel visualization. This could be explained by the low parasitemia on the blood spotted finger prick which yield very small amount of DNA. The binding of the radioactive probe with the specific DNA sequence will increase the visual detection of positive samples. Major reasons for not adapting modern

molecular biology tools under field conditions include; non practical feasibility where electricity and fridge are essential to be found, the transportation of the required equipments (PCR, centrifuge and others) in the long way that is usually harsh will expose these instruments to breaking. The high cost of equipments, instruments and reagents will make the cost effectiveness for using these tools more difficult. In the other hand, the utility of these powerful tools are more useful and practical if used by academic and ministry of health to assist in molecular epidemiology of diseases creating major public health problems and assist in preventive control plans and strategies.

### CONCLUSION

Although, the PCR is known to be highly sensitive and specific tool in disease identification and typing but hybridization technique proved to be more sensitive.

*L. donovani* isolates from Sudan are genetically diverse. An emerge of Sodium stibogluconate, (the main drug used to treat cases) resistance is noticed (Croft *et al.*, 2006; Khalil *et al.*, 1998). The role of sub-clinical cases in disease transmission. All these elements need further studies to identify a potent molecular tools that can be used on clinical samples and provide useful clinical and epidemiological information of the disease and help to identify new mutations that affect disease clinical presentation and response to treatment (Barry and Miriam, 2006).

### REFERENCES

- Abdalla, N.M., Z.A. Hilmy, O.F. Osman, N.S. Daifalla and M.M. Magzoub, 2001. Prolong Serological and molecular study on sub-clinical leishmania focus in the Nuba Mountain, Western Sudan. 50th Anniversary of The American Society of Trop. Med. And Hy. Nov. 2001.
- Abdalla, N.M., M.E. Ibrahim, A.M. El-Hassan, O.F. Osman and N.S. Daifalla *et al.*, 2002. Molecular epidemiology and clinical study on leishmaniasis in Nuba Mountain-Sudan. Acta Parasitol. Turcica, 26: 23-30.
- Barker, D.C., 1987. DNA probe diagnosis of human leishmaniasis. Parasitol. Today, 3: 177-184.
- Barry, G.H. and B. Miriam, 2006. Phylogenetic analysis as a tool in molecular epidemiology of infectious diseases. Ann. Epid., 16: 157-169.
- Croft, S.L., S. Sundar and A.H. Fairlamb, 2006. Drug resistance in leishmaniasis. Clinic. Microbiol. Rev., 19: 111-126.
- El-Harith, A.E., A.H. Kolk, P.A. Kager, J. Leeuwenburg, R. Muigai, S. Kiugu and J.J. Laarman, 1987. Evaluation of a newly developed direct agglutination test (DAT) for serodiagnosis and seroepidemiological studies of visceral leishmaniasis comparison with IFAT and ELISA. Trans. R. Soc. Trop. Med. Hyg., 81: 603-606.
- El-Hassan, A.M. and E.E. Zijlstra, 2001. 2001. Leishmaniasis in Sudan. Transact. R. Soc. Trop. Med. Hygiene, 95: 27-58.
- El-Safi, S.H., W. Peters, B. El-Tom, A. El-Kadarow and D.A. Evans, 1991. Studies on the leishmaniasis in Sudan. 2- Clinical and Parasitological studies on cutaneous leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg., 85: 457-464.
- Khalil, E.A., A.M. El-Hassan, E.E. Zijlstra, F.A. Hashim, M.E. Ibrahim, H.W. Ghalib and M.S. Ali, 1998. Treatment of visceral leishmaniasis with sodium stibogluconate in Sudan: management of those who do not respond. Ann. Trop. Med. Parasitol., 92: 151-158.
- Kirk, R. and M.H. Sati, 1940. Studies in leishmaniasis in the Anglo-Egyptian Sudan. II. The skin and lymph glands in kala-azar. Trans. R. Soc. Trop. Med. Hygiene, 33: 501-506.
- Mohammad, A.O., M.R. Naseh, H. Mallorie, J. Ezat-Aldin and R. Yavar *et al.*, 2009. Development of species-specific PCR and PCR-restriction fragment length polymorphism assays for *L. infantum*, *L. donovani* discrimination. Experimental Parasitology, 122: 61-65.
- Neave, S.H.M., 1904. Leishmania donovani in the Sudan. Bri. Med J., 1: 1252-1252.
- Paindavoine, P., E. Pays, M. Laurent, Y. Goltmeyer, R.D. Le, D. Mehlitz and M. Steinert, 1986. The use of DNA hybridization and numeral taxonomy in determining relationship between Trypanosoma brucei stocks and subspecies. Parasitology, 92: 31-50.
- Passos, V.M., O. Femandes, P.A. Lacerda, A.C. Volpini, C.M. Gontijo, W. Degrave and A.J. Romanha, 1986. Leishmania (Viannia) braziliensis is the predominant species infecting patients with American cutaneous leishmaniasis in the state of Minas Gerais, Southeast Brazil. Acta Trop, 72: 251-258.
- Schoone, G.J., G.J. van Eys, G.S. Lighthart, F.E. Tabu, J. Zaal, Y. Mebrahu and P. Laywer, 1991. Detection and identification of leishmania parasites by in situ hybridization with total and recombinant DNA probes. Exp. Parasitology, 37: 345-353.
- Seaman, J., A.J. Mercer, H.E. Sondorp and B.L. Harwardt, 1996. Epidemic visceral leishmaniasis in Southern Sudan: Treatment of several debilitated patients under wartime conditions and with limited resources. Ann. Internal Med., 124: 664-672.

- Siddig, A.M., H. Ghalib, D.C. Shilington, E.A. Peterson and S. Khidir, 1990. Visceral leishmaniasis in Sudan. *Trop. Geogr. Med.*, 42: 107-112.
- Tashakori, M., K. Kuhls, A. Al-Jawabreh, I. Mauricio, G. Schönian, S. Farajnia and M.H. Alimohammadian, 2006. *Leishmania major*: Genetic heterogeneity of Iranian isolates by single-strand conformation polymorphism and sequence analysis of ribosomal DNA internal transcribed spacer. *Acta Trop.*, 98: 52-58.
- Weigle, K.A., L.A. Labrada, C. Lozano, C. Santrich and D.C. Barker, 2002. PCR-based diagnosis of acute and chronic cutaneous leishmaniasis caused by *Leishmania (Viannia)*. *J. Clin. Microbiol.*, 40: 601-606.
- World Health Organization, 1990. Control of the leishmaniasis. Report of a WHO expert committee. *World Health Organ Tech. Rep. Ser.*, 793: 1-158.
- Zijlstra, E.E., A.M. El-Hassan, A. Ismail and H.W. Ghalib, 1994. Endemic kala-azar in Eastern Sudan; A longitudinal study on the incidence of clinical and sub-clinical infection and post kala-azar dermal leishmaniasis. *Am. J. Trop. Med. Hyg.*, 51: 826-836.