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ANTIBODY RESPONSE TO CUTANEOUS LEISHMANIASIS IN SUDAN

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ABSTRACT

The role of antibody levels in protective immunity against cutaneous leishmaniasis (CL) is largely unknown. The aim of this study is to investigate the role of antibody response against CL among Sudanese population. Fifty six clinically diagnosed CL subjects were enrolled in this study. Forty eight of them had healed scar and eight have active lesions. The leishmanin skin test (LST) was 54.7% positive. The frequency of human IL4RP2 alleles was investigated in this study. The results showed 83.9% (183 bp) and 16.1% (253 bp) and the heterozygosity was 39%. The mean antibody levels using direct agglutination test (DAT) has found to be higher against Leishmania major (L. major) antigen than Leishmania donovani (L. donovani) antigen and the difference was found to be significant (P = 0.01 <0.05). The anti L. major IgG was quantified in this study using enzyme linked immunosorbent assay (ELISA). The mean of antibody levels measured by ELISA and DAT was significantly higher in study participants with scars more than those with skin lesions. Overall this outcome would be supportive to indicate a possible role of antibody-mediated protection in CL and would signify the importance of identification of antigens that may elicit protective antibodies.

Keywords: Cutaneous leishmaniasis, *L.major*, *L.donovani*, Antibody response, IgG, Sudan.

Contribution/ Originality

This study is one of very few studies which have investigated a possible role of antibody response against cutaneous Leishmaniasis. Analysis of some immune parameters that related to variation in clinical response should assist in decision-making concerning treatment.

1. INTRODUCTION

Leishmanises are among the Neglected tropical diseases and other diseases of poverty which score highest level of morbidity and mortality. Infection with *Leishmania* parasites causes severe disease in humans. Sudan is considered as one of the most endemic areas of leishmaniasis in the

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world where sharp epidemics involving thousands of people were recorded (WHO, 2007). 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 affected with leishmaniasis is on low incomes and lives in rural areas (Seaman *et al*., 1996).

Disease manifestations are determined predominantly by the host immune response and the parasite species (Murray *et al*., 2005). Host genetic factors probably play an important role in the disease manifestation (Blackwell, 1996). There is no vaccine for leishmaniasis and current therapies are limited by poor efficacy, the requirement for prolonged treatment, and increasing development of clinical resistance (Croft *et al*., 2006).

CL is common in the North and central Sudan. It is characterized by ulcers in the skin that usually self heal, leaving a scar. A major epidemic in mid eighties has occurred in large parts of northern Sudan, causing thousands of cases (El-Safi and Peters, 1991).

The host immunological response has been shown to play a pivotal role in the outcome of *Leishmania* infection. In the absence of a protective type 1 immune response, subjects infected with *L. amazonensis* develop diffuse cutaneous leishmaniasis characterized by nodular lesions with abundant macrophages containing *Leishmania* (Bittencourt *et al*., 1989). Furthermore, host immune mechanisms play an important role in the efficacy of anti-*Leishmania* chemotherapy (Murray, 2001). The level of antibody response appears to reflect the parasite density and the intensity of the infection (Gutierrez *et al*., 1991). However, the role of antibody titers in resolution of CL and protective immunity is largely unknown. Although some studies have shown the advantage of using specific subclass antibodies for the diagnosis of visceral leishmaniasis (VL), only a few reports are available for CL. Analysis of *Leishmania* antigen-specific immunoglobulin isotypes in CL and VL patient sera revealed elevated levels of IgG and IgG subclasses compared to controls (Ravindran *et al*., 2004), (Ozbilge *et al*., 2006). Cytokines elaborated by activated T cells induce the switching of B lymphocytes to several IgG isotypes and are thus obligatory for some humoral responses. IL-4 is among Th2 cytokines that stimulate the production of high levels of IgG (Kotowicz and Callard, 1993). The cure of leishmaniasis depends on the appropriate activation of the parasitized macrophages by IFN-γ secreted by Th1 lymphocytes (Wei *et al*., 1999). IL-4 is one of the natural antagonists of the Th1 immune response which affect the multiplication of *Leishmania* and the evolvement of the infection into the disease (Matthews *et al*., 2000). A significant association of human *IL4RP2* polymorphism with susceptibility to VL has been found in previous studies (Mohamed *et al*., 2003).Information on natural immunity could provide clues to the development of vaccines against CL. Moreover, analysis of some immune parameters that related to variation in clinical response should assist in decision-making concerning treatment. In the present study, the clinical characteristics of CL study subjects have been described. The main objectives are to find out a possible correlation between clinical manifestations and the immunological response investigated in the study subjects using DAT and

ELISA tests. The correlation of human *IL4RP2* polymorphism with clinical and immunological response has also been investigated.

2. MATERIALS AND METHODS

This is a hospital-based study conducted in Giad hospital located in Giad industrial city, Gezira state, a distance of 50km south of Khartoum City. The area has witnessed an outbreak of CL in 2000 (Giad hospital records), where it was a forest land at that time. After which, it has been changed to an industrial area. All study participants or their guardians have signed the informed consent about the purpose of the study.

2.1. LST

A random population survey has been conducted on 137 participants with active and healed lesions. The survey was performed by LST to test the presence of previous CL exposure as indicated by development of cell-mediated immunity. Giad population has not been subjected to LST prior to this study. LST was carried out by intradermal injection of 100 µl of leishmanin suspension containing 5×10^5 /ml *L. major* promastigotes cultured in the laboratory. The administration of leishmanin suspension has induced a local reaction. The induration was measured along two diameters by the ballpoint pen method after 48 or 72 hours. Induration with a diameter of ≥ 5 mm was considered as a positive response (Sokal, 1975).

2.2. Sample and Questionnaire Data Collection

Fifty six study participants with clinical symptoms and signs suggestive of active lesion and/or healed scar characteristic for CL infection were enrolled in this study. The clinical episodes of CL have been confirmed by clinician in the hospital. A questionnaire has been filled for parameters, related to personal information, clinical status, treatment, and infections with other diseases.Aspirate smear from active lesions using a syringe and a needle were collected on glass slides and stained with Giemsa stain to detect the presence of amastigotes using microscopy.

Blood Samples and smear from lesions were further collected on 3MM Whatmann filter paper for DNA extraction and antibody measurement. DNA was extracted from the filter paper samples using the Chelex extraction method (Plowe *et al*., 1995). The DNA extracted was later used to detect the parasite species and to identify human *IL4RP2* polymorphism.

2.3. Amplification of *Leishmania* Parasite Using Primers DB8/AJS3

Specific primer pair DB8 5′-CCAGTTTCCGGCCCCG-3′ and AJS3 5′-GGGGTTGGTGTAAAATAG-3′ have been used in polymerase chain reaction (PCR) to detect *Leishmania* species (Smyth *et al*., 1992). The PCR products size was 700 and 800 bp for *L.major* and *L.donovani* respectively. DNA extracted from blood and smear lesions (50-60 ng/ µl) was used as template. Purified leishmanial DNA of 1 µl (200 ng/ µl) was used as a positive control.

The reaction mixture (25 µl) contained 10× NH_4 buffer, 25 mM $MgCl_2$, 10 mM of each deoxynucleoside triphosphate, 10 mM of each primer, 1 U *Taq* DNA polymerase (Vivantis, Malaysia) and 3 µl of template DNA. Negative control that contains all reagents except template DNA was also included in PCR. Amplification was performed in a thermal cycler (PTC200) Programmed for 37 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, preceded by an initial denaturation for 5 min at 94°C. Final extension was made for 10 min at 72°C. PCR Products and 3 µl of 100 ng/ µl DNA marker were analyzed by electrophoresis in 1.5% agarose gel containing 1 mg/ml ethidium bromide at 85V and photographed under UV illumination.

2.4. Amplification of Human *ILRP2* Polymorphism

The polymorphism *IL4RP2* was amplified using primer pair (5′-TAGGCTGAAAGGGGGAAAGC-3′) and (5′- CTGTTCACCTCAACTGCTCC -3′) (Arai *et al.*, 1989). PCR products for *IL4RP2* repeat is 183, 153 and 123- bp. DNA extracted previously was used as template in this experiment. The reaction mixture (25 µl) contained 10× NH_4 buffer, 25 mM $MgCl_2$, 10 mM of each deoxynucleoside triphosphate, 10 mM of each primer, 1 U *Taq* DNA polymerase (Vivantis, Malaysia) and 3 µl of template DNA. Amplification was performed in a thermal cycler (PTC200) programmed for 37 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, preceded by an initial denaturation for 5 min at 94°C. Final extension was made for 10 min at 72°C.

2.5. DAT

The antibody response against *Leishmania* promastigotes crude antigen was measured in 56 study participants using DAT. Antigen has been prepared from a culture of *Leishmania major* and *Leishmania donovani* strains according to the modified procedure (Harith *et al.*, 1995). The final concentration of the prepared antigen was 5×10^8 promastigotes/ml stock antigen. A punch (6 mm) of the filter paper blood sample was incubated overnight into a 1.5 ml eppenderof tube contained 200µl of sodium normal saline at room temperature.

The DAT was performed on 96 V-shaped microtitre plate as described by (Harith *et al.*, 1995). The result was read visually against a white surface. A clear titer of >3200 was considered positive for the test and indicative for the disease. A titer of ≤1600 was considered seronegative.

2.6. ELISA

A puch of around 6 mm of blood sample on filter paper was incubated for 1hour in 56°C in a 1.5 ml eppenderof tube. After that, 500 µl of phosphate buffer saline (PBS), 0.05% Tween and 0.5% BSA was added and incubated for 2hours at room temperature on a shaker (Harith *et al.*, 1988). The samples were then vortexed for a few seconds and stored at -20°C. Antigen for ELISA was obtained from a culture of *L. major* promastigotes and prepared (Yoshie Do Rosário *et al.*,

2005). The final antigen concentration was 1,000 µg/100 ml. A positive control was prepared as a pool from the samples sera. Negative controls well contain no sera.

ELISA was performed to measure the level of anti-*L.major* IgG among the study participants (Quinnell *et al*., 1997). Spectrophotometric reading of the plates was made using ELISA machine (RFE 51118300). The average absorbance values at 492 nm were calculated for each set of positive control, negative control and samples. The numbers of IgG antibody units were calculated from the OD value for each sample. Antibody concentrations were expressed in arbitrary units, relative to a highly positive control serum titrated on each plate. Two cut-offs were chosen to define seropositives: (i) the best cut-off estimated from the frequency distribution of antibody titres and (ii) the mean-3 standard deviation of the negative control samples (Quinnell *et al*., 1997).

2.7. Statistical Analysis

Data was analyzed using SPSS version 16 (Stata Corporation, http://www.stata.com). The anti *L.major* IgG titer was tested for normality using the Kolmogorov-Smirnova test. Differences were considered statistically significant if $P \leq 0.05$. Differences in sample means were evaluated using both parametric and non-parametric approaches where appropriate. The Student's t-test was used for pairwise comparisons. Bivariate comparisons were examined using Pearson rank correlation coefficients (r) and values were corrected for duplicates. Two-tailed significance values were used.

3. RESULTS

3.1. LST

The induration of the LST reaction size was detected in the range from 5-16mm. LST was considered positive in 55.6% of the participants. The number of positive LST participants was found to be increased with age (df = 2, $P = 0.00 < 0.05$) (Figure 1).

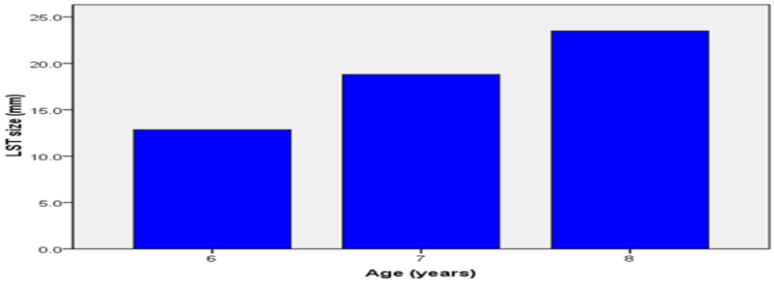


Figure-1. Variation of LST size with age. LST:Leishmanin skin test; x-axis represent age of random samples from school children born in Giad city, y-axis represent the size of the induration developed after administration of leishmanin suspension.

3.2. Questionnaire and Clinical Data Results

The assessment of the survey using a questionnaire revealed that all the study participants were originally coming from Leishmaniasis free areas and there was no significant difference among the study participants in ethnic groups, types of houses, a place of sleep, and the use of the bed nets. Differences in sample means were evaluated by T-test and One way ANOVA.

Infections with other diseases has not been detected among the study participants in the present study specially malaria and helminthes infection.

Different clinical manifestations of CL among study participants including scar, skincrease, ulcerated lesions, clustering (multiple scars on the same site), nodules, ulcers and pus were observed.

The CL skin scar and lesions were most frequent on the exposed parts of the body, particularly the limbs. The face is less frequently affected. The general appearance in active lesion CL participants was nodules, ulcers and pus represented by 14.2%. The general appearance in recovered CL participants was the scar and skin crease represented by 85.7% (Figure 2). However, 55% of the participants have shown multiple lesions on different sites (Figures 3&4) and 20% have shown clustering. Participants with fever, allergies and papules have not been observed during this study.

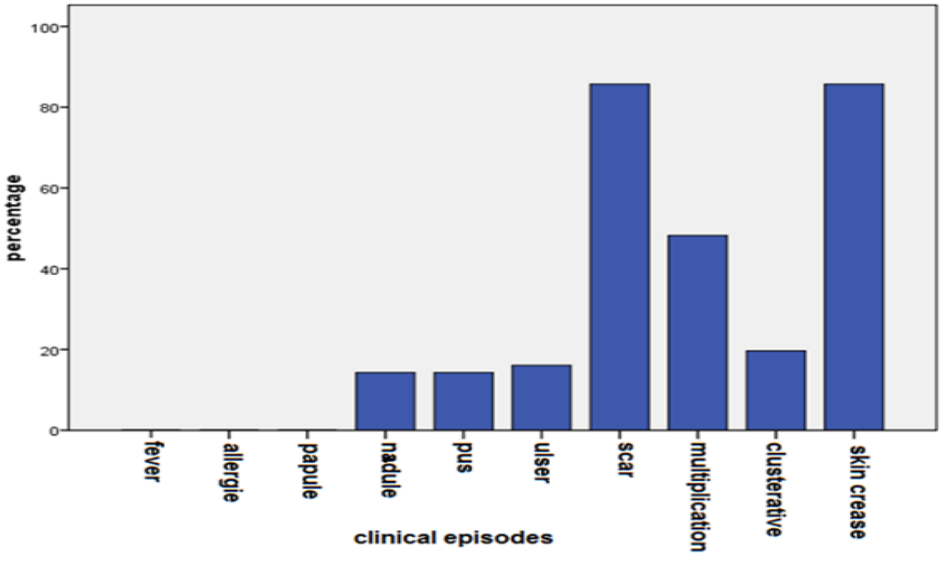
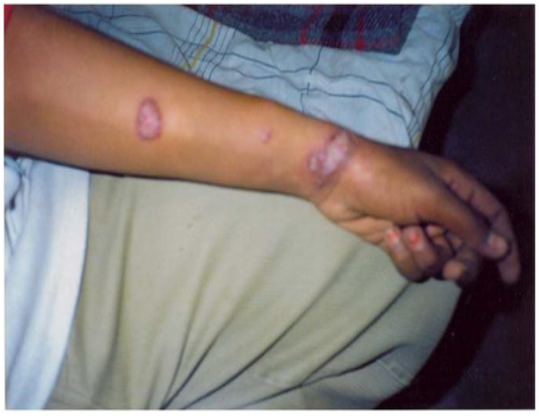


Figure-2. The percentage of clinical episodes developed by CL study subjects. CL: cutaneous leishmaniasis, 85.7% of the general appearance in recovered CL participants was represented by the scar and skin crease



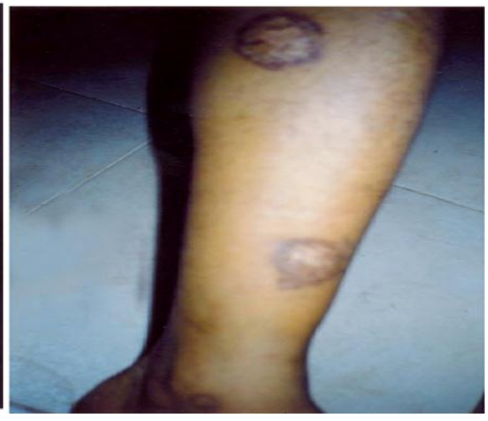


Figure-3. CL nodules with active multiple lesions on one limb. **Figure-4.** CL scar of healed multiple lesion on one leg.

A weak to fair relation between age and the general appearance was detected using Pearson Correlation (r = 0.343, P = 0.010). This indicates that 34% of the variation in the general appearance was interpreted by the variations in age.

A significant variation of the general appearance between males and females has been found in this study using paired samples test (t = 8.804, P = .000< 0.05) and Independent samples Mann-Witney U-test (P = .031< 0.05).

Most of lesions that have been observed during this study have healed spontaneously. Study participants have been observed to use medical antifungal treatment or traditional treatment to accelerate lesion healing. Others have been noticed burning their lesions. Those who did not receive any type of treatment have the longest duration of infection, while those who had burned their lesions or used medical treatment have a lower duration of infection. The correlation between duration of infection and type of treatment was found statistically significant, using the One Way ANOVA test (P = 0.037 < 0.05) (Figure 5)

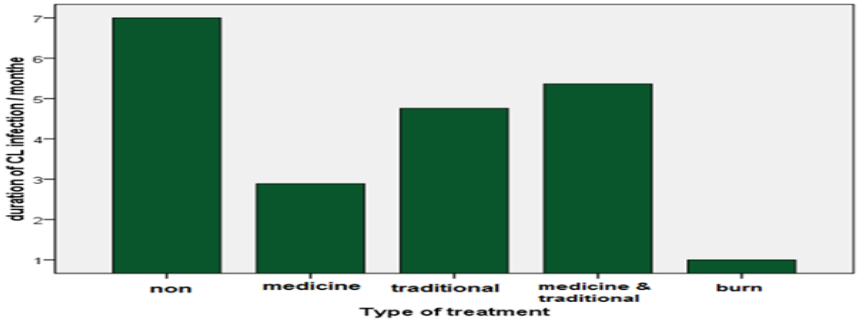


Figure-5. The correlation between duration of infection and type of treatment. Those who did not receive any type of treatment have the longest duration of infection, while those who had burned their lesions or used medical treatment have a lower duration of infection.

The variation in treatment type has found to have a significant effect on the appearance using paired samples test (t = 6.028, P = 0.000). The majority of participants who classified as recovered were shown to use medical treatment.

Leishmania parasite couldn't be detected in peripheral blood or in smear lesions using both microscopy and PCR.The frequencies of human *IL4RP2* alleles 1(183 bp) and 2(253 bp) are 0.92, and 0.58 respectively (n= 56) with *IL4RP2* alleles positive on 98.2%, and the heterozygosity is 0.39 (Figure 6).

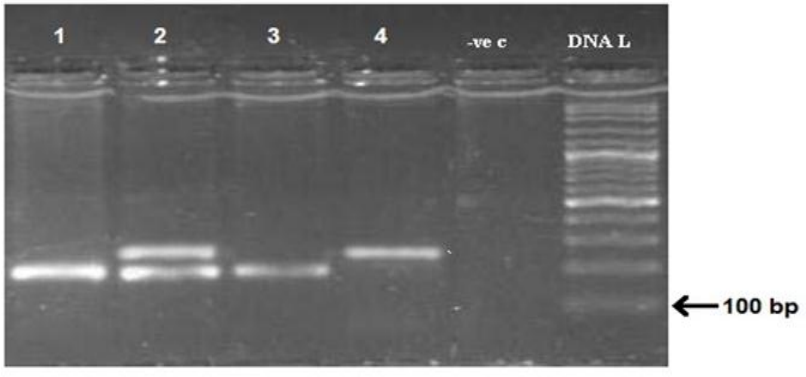


Figure-6. Amplified DNA of *IL4RP2* gene isolated from peripheral blood of CL subjects. Lanes 1and 3 is homozygous for allele1, lane 2 is heterozygous for alleles 1and 2, lane3 is homozygous for allele 2, -ve c: is a negative control, DNA L is 100 bp

It has been found that there is a significant correlation between *IL4RP2* polymorphisms and the general appearance (χ^2 = 64.509, P = .000< 0.05) using Chi square test. Allele 1 was more dominant among recovered participants (with scar and skincrease) compared to those with active lesion.

Results of the DAT test demonstrated a significant variation in the magnitude of the antibody response against *L.major* and *L.donovani* antigen with higher levels detected against the *L. major* antigen using independent samples T-Test, (P = 0.001 <0.05) and Leven test (P = 0.02 <0.05).

DAT was positive in 82.1% of sera samples detected with *L. major* antigen. The range of titer reading was 100-25600 (mean = 8752). This refers to the existence of variation with 37.5% had a titer of 6400. DAT was positive in 76.8% of sera samples detected with *L. donovani* antigen. The range of titer reading was 100-25600 (mean = 5529) with 39.3% had a titer of 3200.

Further analysis have illustrated that the age groups 11–20 and 21-30 years have shown a higher mean of DAT titer than other age groups (Figure 7).

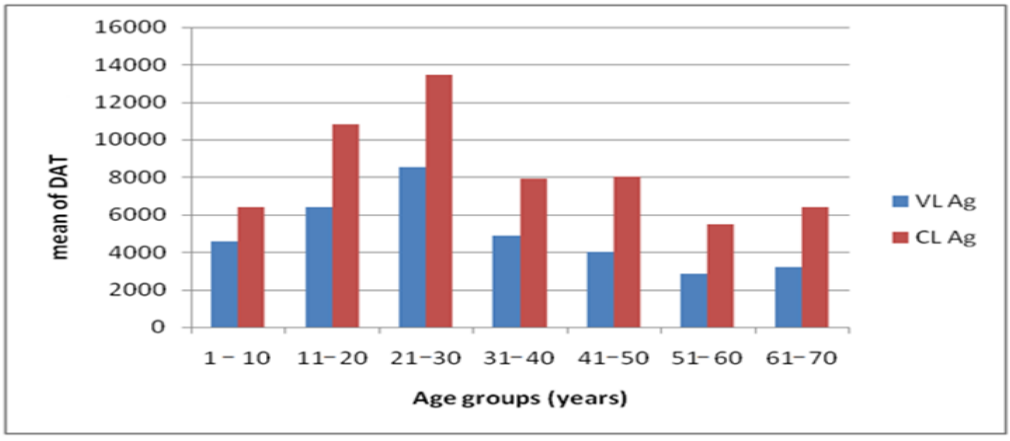


Figure-7. Variation of the mean of anti- *L.major* and anti-*L.donovani* antibody level with age. Age groups 11–20 and 21-30 years have shown a higher mean of DAT titer than other age groups, DAT: direct agglutination test, mean of DAT: mean of DAT titer, VL Ag: *L. donovani* antigen, CL Ag: *L. major* antigen.

The level of the mean of antibody was higher in females compared to males, with ± 95% confidence interval, and was statistically significant T. test, ($P = 0.00 < 0.05$) (Figures 8 & 9).

Figure-8. Variation of the mean of anti- *L.major* antibody level with gender. DAT titer was higher in females compared to males. DAT level: Direct agglutination test titer, *L.major* Ag: *L.major* antigen.

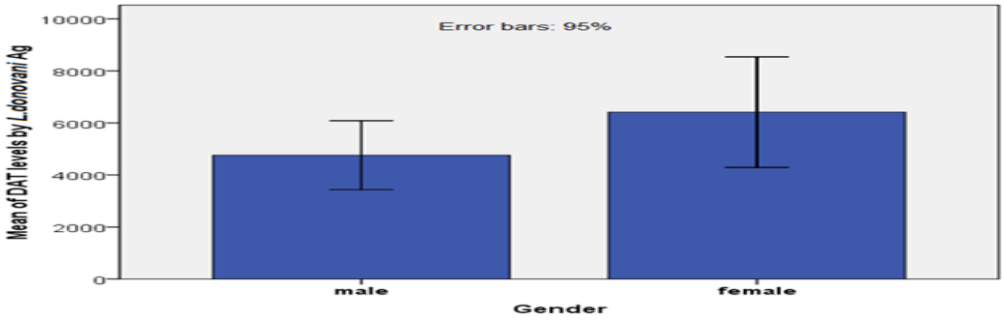


Figure-9. Variation of the mean of anti-*L.donovani* level with gender. DAT titer was higher in females compared to males. DAT level: Direct agglutination test titer, *L.donovani* Ag: *L.donovani* antigen.

The effect of *IL4RP2* polymorphisms on the level of anti-*L.major* antibody and anti *L. donovani* antibody was significant using paired samples test (t = 11.567 and 9.269 respectively and, *P* for both = .000).

The anti-*L.major* IgG levels were found normally distributed among the study participants. The range of measurements of anti-*L.major* IgG concentration was (7187.8 - 13101.81 IU/ml). This refers to the existence of variation and the mean was 9607IU/ml**.**

The antibody level showed an increase with age but with differing patterns. Anti-*L.major* IgG level showed almost a liner increase with age lower than 20 years. However, it was stable in all age groups, and was highest in 20-30 years (Figure 10).

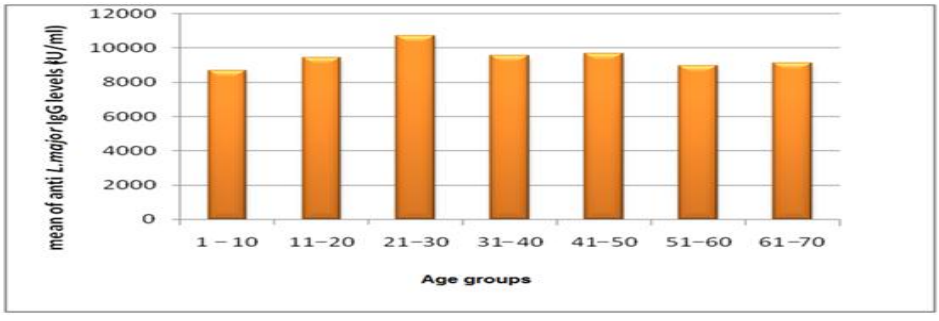


Figure-10. Variation of the mean of anti- *L.major* IgG level with age. IU/ml: International units per ml.

The level of the mean of anti- *L.major* IgG level was the same in males and females, error bar represent the ± 95% confidence interval, and was statistically not significant (*P* = 0.9).

The effect of *IL4RP2* polymorphisms on anti-*L.major* IgG level was highly significant as shown by paired samples test (t = 54.736, *P* = .000). . Allele 1 (183 bp) was more dominant among participants with high titer.

The anti-*L.major* IgG level has a highly significant effect on infection status (t = 54.736, *P* = .000), while relatively significant effect have been detected for the effect of anti-*L.major* antibody and anti-*L. donovani* antibody on the infection status (t = 11.567 and 9.269 respectively and, *P* = .000).

The mean of anti-*L.major* IgG and anti-*L.major* antibody levels were higher in subjects with healed scar compared to those with active lesions. Anti-*L.donovani* antibody level has shown less values (mean = 5528.57) compared to anti-*L.major* IgG (mean = 9569.97) and anti-*L.major* antibody levels (mean = 8751.79). Moreover, the mean of anti-*L.donovani* antibody level was higher in partcipants with active lesion compared to those with healed scar.

4. DISCUSSION

The present study demonstrated that Giad population has been exposed before to *Leishmania* infection as investigated by LST. A higher proportion of positive LST was obtained in older age

group than in the younger one. One possible explanation is that older groups were more likely infected through occupational exposure than younger groups as has been suggested before (De Yarbuh, 1997).

CL skin scar and lesions were mostly found during this study on the limbs in particular the lower parts. This often reflects the clothing habits of the study participants particularly at night. The same observation was previously made in different areas (Mohamed, 1997). The present results could also be explained by the difference in height. Sand flies are supposedly flying only short distances; very low and moving along the ground often in short jumps (Killik-Kendrick *et al*., 1986; Doha *et al*., 1991; Alexander and Young, 1992).

A potential role for environmental factors has been observed during this study. It has been found that 34% of the variation in the general appearance was interpreted by the variations in age. Age effects on scar number have been observed before (Davies *et al*., 1997) possibly because of an intrinsic age-related change in immune status.

A significant variation of the general appearance between male and female with males more affected than females. This is again reflects clothing habits difference between males and females beside males are more likely to be exposed to sandfly than females. However, no significant effect of gender on clinical symptoms was observed in previous study (Davies *et al*., 1997).

Cutaneous leismaniais is characterized by antibody response that last for short period after infection however, there was no significant difference detected in antibody response between study participants who had the infection one month ago and those having the infection for the past three years.

Significant variation of the infection status was detected with anti-*L.major* and anti-*L. donovani* antibody levels. The mean of anti-*L.major* antibody and anti-*L.major* IgG levels were higher in participants with healed scar compared to those with active lesions. Moreover, the mean of anti-*L.donovani* antibody level was higher in subjects with active lesion compared to those with healed scar. These results could illustrate the specificity of the anti-*L.major* antibodies in disease progression and at the same time the possibility of development of cross immunity against homologous species. Complete immunity to the homologous species and strains of *Leishmania* is thought to be established after infection. Cross immunity may sometimes develop due to multiple parasite host interaction that involves all the defense components of cellular and humoral immune system (Handman, 2001).

The mean of antibody levels was found to be higher with *L.major* antigen compared to the mean of antibody levels by *L.donovani* antigen in different age groups. The antibody level showed an increase with age but with differing patterns. Anti-*L.major* IgG level showed almost a linear increase with age lower than 20 years. However, it is stable in all age groups, and highest in 20-30 years. The immune system changes throughout life. At birth, specific immunity is not fully developed. However, newborns have some antibodies, which crossed the placenta from the mother

during pregnancy. These antibodies protect newborns against infections until their own immune system fully develops.

The present study has pointed out a possible role of IgG as diagnostic marker for diseases progression. Anti-*L.major* IgG levels were higher in subjects with healed scar compared to those with active lesions. On other hand, previous results showed that there were increased levels of IgG in participants with active CL which appear to be opposing to the data obtained from the present study (Ozbilge *et al*., 2006). In murine models, it has been shown that IgG not only fails to provide protection against *Leishmania* parasite, but it actually contributes to disease progression (Miles *et al*., 2005).

In the light of these findings, it should be possible to conclude that in human CL, measurement of IgG level in sera could be used as a diagnostic or prognostic marker to evaluate subjects with active CL. To ascertain this, detailed studies include IgG total and IgG subclasses levels with a higher number of cases need to be planned.

It has been found that there is a significant correlation between *IL4RP2* polymorphisms and the general appearance of the study participants. Allele 1 (183 bp) was more dominant among recovered participants (with scar and skincrease) compared to those with active lesion.

The effect of *IL4RP2* polymorphisms on the level of anti-*Leishmania* antibody and on anti-*L.major* IgG level was found to be significant in the present study. Allele 1 (183 bp) was more dominant among participants with high titer. Whether this polymorphism could be used as an indicator for the disease progression will need further investigation.

IL-4 level has been shown in previous studies to distinctly correlate with levels of parasite burden in various clinical forms of CL (Kumar *et al*., 2009). Differences in pattern of IL-4 production upon stimulation with *L. donovani* and *L. major* antigens has previously been detected (Kurtzhals *et al*., 1995).

5. CONCLUSION

This study has suggested that infection with CL leads to a significant elevation in the levels of antibodies and in the level of anti-*L. major* IgG antibodies. Antibody response can be obtained for *L. major* antigen and *L. donovani* antigen indicating a cross reactivity. Immunological response can last for long period of time even after recovery. Furthermore this study showed that human *IL4 RP2* polymorphism may play a role in susceptibility to CL patients. The small numbers of cases limited the power of the study to evaluate this variable.

Authors' contributions: HMA conceived and designed the study, coordinated field and lab works, participated in data analysis, interpretation of data, drafted and revised the manuscript; MIA conducted the field work, collected the samples, performed the lab experiments, analyzed the data and helped in drafting the manuscript; AME given critical comments to study design and revised the manuscript; OFO participated in the study design and lab experiments; HSM

participated in field work and design of lab experiments;. All authors read and approved the final manuscript. HMA and HSM are guarantors of the paper.

6. ACKNOWLEDGEMENT

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Conflict of interest: None declared

Ethical approval: Ethical approval was obtained from Faculty of Science, University of Khartoum, (Khartoum, Sudan).

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