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Serological Diagnosis of Cutaneous and Visceral Leishmaniasis

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Abstract

Upon deployment into war-torn endemic countries, military personnel become vulnerable to local infections. Due to the absence of preventive vaccines, we examined such situations with regard to leishmania infections. Serum samples from exposed subjects were tested by ELISA for the presence of antibodies specific to cutaneous and visceral leishmaniasis. A specific ELISA assay was constructed using Exoantigens released from cultured promastigotes. Indirect antibody levels were detected using monoclonal anti-human IgG antibody as the target, where an absorbance of 0.3 was used as the cut off. Serum was tested by ELISA initially at 1/100 dilution and further diluted and tested to determine the end-point. We report antibody profiles of cutaneous leishmania cases reported from Belize, Afghanistan, Pakistan, Iraq, and South American countries such as Peru, Ecuador, Bolivia and Guyana, Brazil, and other parts of endemic areas. The cutaneous form showed very high antibody level with end-point dilution of 1/1600. Circulating antibodies were detected in clinical cases of *Leishmania brasiliensis*, *L. mexicana*, *L. viania*, *L. amazonensis*, *L. major and L. tropica* suggesting that antibodies can be used as significant diagnostics. Only a few cases of visceral leishmaniasis from Malta were reported; serum was tested at 1/200 and found to be positive. High levels of antibody were detected, as opposed to previous observations where antibodies against cutaneous leishmania were either nonexistent or undetectable. Detected antibody levels were quantified and end-point determinations were made for ELISA. The antibody response generated is of very high titer and can be detected by ELISA. Specific antibodies against cutaneous and visceral leishmaniasis can be used for diagnostic purposes and results are available within few hours. Immunological testing is far quicker than the parasitology cultures and can easily be performed under field conditions, making it an ideal diagnostic for leishmaniasis.

INTRODUCTION

Historically, tropical diseases have been a significant threat to military operations in tropical zones [1]. In recent years, military deployments to Afghanistan and Iraq for peace keeping purpose have placed large numbers of military personnel in these countries [2]. British troops have also gone to Belize for jungle training [3]. Even short periods of stay in the local environments of endemic countries is enough to receive bites from insect vectors and be exposed to Leishmania infection, which is potentially dangerous for military personnel, travelers and visitors alike. There is every chance of contracting the infection by the bites of sand fly vectors. Leishmania infections in humans, caused by 25 different species, are spreading due to several factors. Currently, leishmaniasis shows a wider geographic distribution and increased global incidence of human infections than previously known. Current statistics indicate that nearly 1.5 million new cases are reported in the cutaneous form (CL) from seven countries (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria) and 90% of visceral form (VL) occurs in rural and suburban areas of five countries (Bangladesh, India, Nepal, Sudan and Brazil) [4]. The infection can be treated with drugs, but there is no prophylactic medicine or protective vaccines against leishmania parasites currently available. This paper deals with the exposure and clinical features of leishmaniasis cases encountered in British veterans deployed to serve in different endemic regions. Every year, many Britons visit overseas countries and, as expected, many cases of imported malaria, leishmania, filarial and other infections are recorded. Reported cases of leishmaniasis are scattered in the literature and there is no comprehensive report on the potential dangers of leishmania infection in Army veterans and travelers to potentially dangerous endemic regions.

Hepburn et al. [5], reported cutaneous lesions in British troops deployed into Belize; out of 306 soldiers subjected to leishmania infection, 187 cases were confirmed parasitologically, samples from 117 cases yielded culture positive results and 70 cases were positive for leishmania bodies in biopsy specimens. In their report, they have mentioned that serology is not helpful for cutaneous disease because antibodies tend to be undetectable or present in low titer [5]. Parasitological examination was an option for testing the exposure to leishmania infection in these cases. Altogether there were 78 cases of *L. b. brasiliensis* and 29 cases exposed to *L. m. mexicana*. No muco-cutaneous lesions were recorded from these cases. No serology was investigated; however, clinical-pathology has been done on some of these cases [6]. Subsequently, an additional 150 cases caused by *L. brasiliensis* have also been reported by Freeman et al. [7], in British troops serving in Belize. Knowledge of this disease is of particular importance to the Military Medical Officers, as avoidance of infection under duty of care is not possible. In 1994, pathological analysis of CL cases in British troops was described from Belize reporting 34 cases [5]. Recently, Malik et al. [8], reported 39 cases of imported VL over a duration of 1985 to 2004 in the civilian populations as reported in Hospital for Tropical Diseases in London; 55.5% of patients had been to tourists to different endemic areas and 44.5% were immigrant refugees from endemic countries. A variety of tests such as IFAT, DAT, latex agglutination, and rk39 serology tests were performed on 33 patients. This paper deals with HIV positivity and relapses after chemotherapy treatment. But intensity of serological responses due to leishmania is not available.

Recently, there was an imported CL case of a traveler who visited Belize, returned to Northern Territory in Australia, and subsequently travelled to England. In addition, an Australian tourist has contracted cutaneous infection while on a tour into Belize. Parasitological examinations revealed *L. brasiliensis* and *L. mexicana* infections [9]. There was an additional report of 8 positive CL cases acquired from Belize, which were successfully treated with ketoconazole [10]. Visits of foreign people into the endemic regions certainly allow them to be exposed to due to prevalence of sand fly bites. Depending upon their immune status, Leishmania infection presents quickly in immune-compromised individuals or become occult infection with delayed onset. Many publications deal with infection of leishmania but do not investigate the sero-conversion and antibody profile.

In this paper, we examined the utility of serology as a diagnostic tool. Serum was tested in blind to demonstrate the specific circulatory antibody response against Leishmania parasites. Once the serology results were available, the patient profile (in other words serum samples) were revealed. The immunological response in the form of detectable antibody levels is explored and seen as predictive for exposure to infection. Antibody response as a diagnostic tool is later examined and correlated with parasitological findings. The combination of parasitology and serology are taken together to describe the exposure and clinical entity of leishmaniasis. Samples were tested retrospectively are from British Army personnel and also citizens as visitors to different endemic regions (Belize, Afghanistan, Pakistan, Brazil, and Malta). It further confirms that military personnel and endemic visitors are being exposed to Leishmania infection. This report covers serum samples from 50 cases of which 18 samples were from British Army veterans, with remainders were from travelers. The remaining 32 samples were submitted to the Liverpool School for Clinical Examinations.

MATERIALS AND METHODS

Patient clinical material

A total of 50 clinical patient samples formed the basis of this study. Returning veterans showed some evidence of clinical symptoms ascribed to leishmania infection. There were skin lesions in case of cutaneous form of infection and liver and spleen involvements with visceral cases. There were more cutaneous forms of infection than visceral involvement. The clinical materials were referred to the Liverpool School of Tropical Medicine for diagnostic purposes. Skin snip as well as spleen biopsy materials were collected under sterile conditions and all biopsy materials were processed. The materials were cultured for development of promastigote stages. The cultures were set up in NNN media, as well as in Schneider medium supplemented with 20% FCS. Parasites species were identified based on isoenzyme analysis [9]. Serum samples were prepared from the blood samples and tested for determination of antibody response against Leishmania parasites. Each serum sample has been coded at the Liverpool School of Tropical Medicine and was made blind while performing the antibody detection ELISAs.

Antibody detection ELISA

Antibody detection ELISAs were constructed by using the released antigens of promastigote stages of Leishmania parasites as the capture antigen for Leishmania specific antibodies that were present in serum samples [10,11]. These antibody detection ELISAs were manufactured at Cellabs Pty Ltd, Brookvale NSW, Australia. In particular, promastigotes of Leishmania species were maintained *in vitro* under sterile conditions in serum-free-media for 2-3 days at 37°C. The released antigens in the serum-free media were harvested and labeled as exo-antigens of a particular Leishmania species. The exo-antigens were measured by spectrophotometer and quantified. ELISA wells werecoated with Exoantigens from *L. donovani* and *L. infantum* promastigotes and are classified as visceral leishmania ELISA. ELISA plates coated with Exoantigens from *L. tropica, L. mexicana* and *L. panamensis* promastigotes are included as cutaneous leishmania ELISA. Then appropriate reagents were used for preparing the ELISAs for testing serum samples from visceral (VL-Antibody Detection ELISA) and cutaneous (CL-Antibody Detection ELISA) infections, respectively. It was found that an appropriate optimal dilution of serum samples is required for demonstrating the antibodies react against visceral and cutaneous forms of infection. Serum samples were initially diluted 1/200 for detection of antibodies against the visceral forms and 1/100 for detecting antibodies against the cutaneous forms. Diluted serum samples were incubated in ELISA wells coated with respective exo-antigens for 60 min at 37°C. Then, wells were reacted with monoclonal anti-human IgG–HRP conjugate and chromogen substrate (TMB) was used to develop color which is reciprocal to the amount of bound antibodies. Plates were read in dual wavelength (450/620 nm) and absorbance readings were plotted [10,11]. The serum samples that were found to be very strongly positive in the initial

tests were further serially diluted two-fold for demonstrating the end-point titrations.

Additional tests

Some of serum samples that were derived from visitors to the endemic region were also tested for potential exposure to malaria infection. Additional ELISA tests were performed for detecting antibodies against malaria by using Pan Malaria Antibody ELISA kit, and also for active malaria infection by antigen detection ELISAs (histidine rich proteins as well as pLDH antigen). These kits were also manufactured by Cellabs Pty Ltd.

RESULTS

Processing of biopsy material and parasitological culture

Biopsy materials Figure (5) were cultured in NNN medium and Leishmania parasites were identified based on parasitological analysis, as well as by the isoenzyme analyses. Based on identification, they were speciated and the Leishmanias pecies encountered are listed in (Table 1).

Antibody detection in serum samples

Altogether, a total of 50 serum samples derived from individuals who visited Belize and other countries Table (1) were tested for presence of Leishmania specific antibodies. An indirect immune fluorescence staining was performed on promastigotes revealed the specific staining of leishmania parasites (Figure 5). The samples came from individuals who have exposed to Leishmania infection during their brief visit to the endemic regions,of which there were 14 samples from returning veterans from Belize. Table (1) shows specimen details. Those samples which were parasitologically positive were taken up for further analysis and were subjected for antibody testing.

Serum samples from Belize

Figure (1) shows the antibody levels in 14 samples when tested at 1/100 dilutions. Except one (WB 413), all other 13 samples showed absorbance >0.8 and of these 7 samples (*L. mexicana* –WB320, 319, 318 424; *L. brasiliensis* WB 324, 322, *L. viania* WB325) showed antibody levels >2.0 absorbance indicating that very high level of antibodies are generated during cutaneous leishmania infection. Further titrations were done starting from 1/200 to 1/3200 dilution, and the data are presented in Figure (2). The pattern of titration showed a similar trend in all samples showing the end-point positivity at 1/800 dilution, however, the sample WB 418 registered higher absorbance values (OD=1.077) at 1/800 dilution. At a further higher dilution (ie, at 1/1600), only one sample (WB 417) showed elevated absorbance (OD=0.738) higher than the cut off (OD=0.3).

Serum samples of returning veterans from Afghanistan, Pakistan and Iraq

There were n=10 parasitologically positive samples from veterans who had been to Afghanistan, Pakistan and Iraq and were infected with *L. tropica* and *L. major*. A sample from Iraq was just marginally positive (OD=0.39), whereas remaining 9 samples from Afghanistan and Pakistan showed a very high antibody response (OD >1.0) and were definitively positive. Of these, 6 samples showed OD>2.0 Figure (3) at 1/100 dilution.

Table 1: Sample history: n=50 clinical specimens collected from individuals who visited the following countries under duty-of-care.

Endemic region (Country)	No of Samples	Parasitological Observation (number in bracket indicated the no of isolates/samples)	Clinical observation (if any)	Year of Incidence and Antibody Determination and other information
Belize	14	*L. brasiliensis* (6) *L. mexicana* (4) *L. viania* (4)	Skin lesion noticed	Incidence in the years 1999-2009 Antibody detected. Very high with endpoint dilution of 1/1600
Afghanistan	4	*L. major* (4)	Skin lesion	Incidence in the year 2004. High level of antibody response against *L. major* has been reported.
Pakistan (1+) Unknown (2)	1+2	*L. tropica* (3)	Skin lesion	Incidence in 2007 (Pakistan). Very high level of antibody response against *L. tropica*.
Iraq (1) + Unknown (2)	1+2	*L. major* (3)	Skin lesion	Incidence in the year 2007. Relatively low level of antibody response seen.
Peru (2) Bolivia (1) Guyana (1) Panama (1)	2+3	*L. brasiliensis* (5)	Skin lesion	Incidence in 2000-2005 (Peru), 2003 (Bolivia), 2004 (Guyana), 1968 (Panama). High and low level of antibody response reported.
Brazil (1)	1	*L. amazonensis* (1)	Skin lesion any muco-cutaneous forms?	Incidence in 1994. Low antibody response seen
Malta (1) Mediterranean (1)	2	*L. donovani* (2)	Visceral form splenomegaly Biopsy results	Incidence (1994-2009) visited twice. Preponderance of infection along with high antibody response.
Peru, Oman, Morocco, Malta	4	One sample each unknown species		Incidence in 2000-2005 (Peru), 1998 (Oman), 1997-2009 (Morocco), in 1998 (Malta). Antibody response is significant.
Unknown places	14	Undefined species	Leishmania parasites isolated	Antibody response is high.

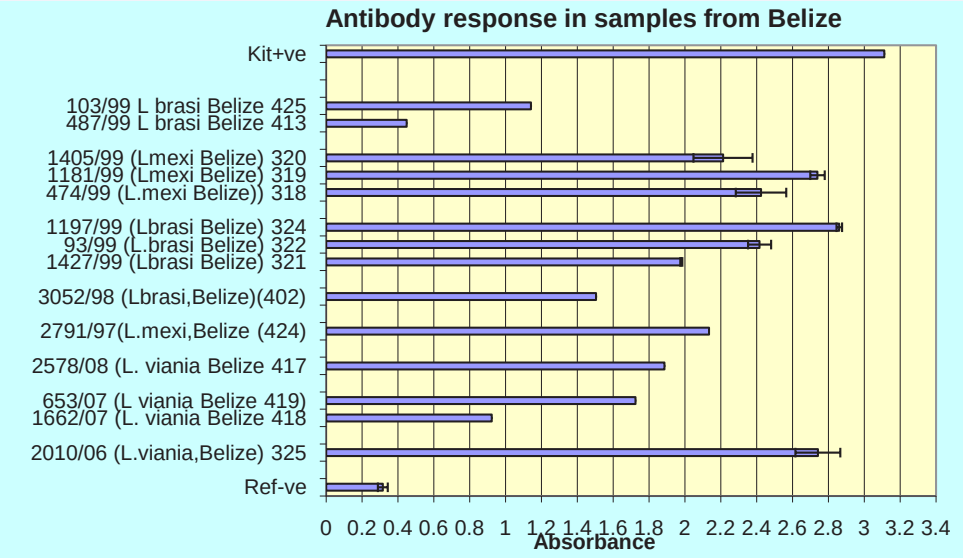

Figure 1 Antibody response seen in serum samples from Belize.

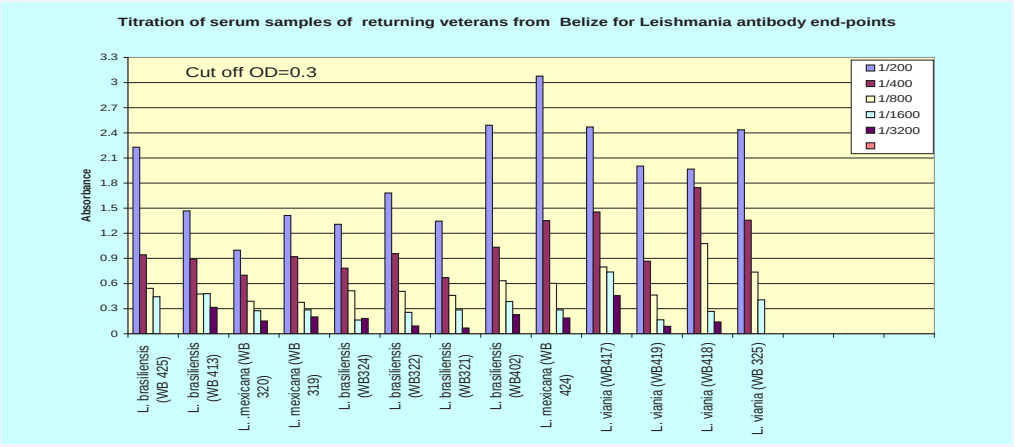

Figure 2 Titration of serum samples derived from different *Leishmania* species

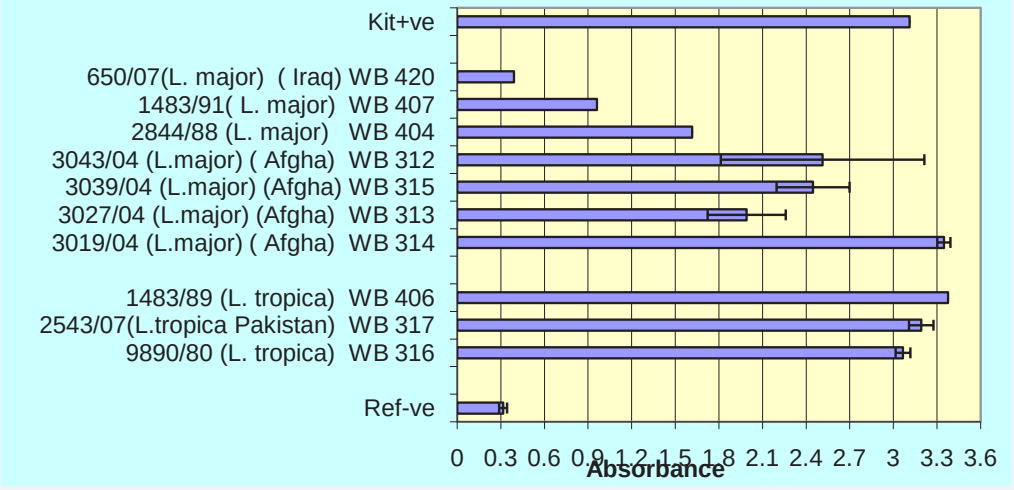

Figure 3 Antibody levels in *L. tropica* and *L. major* infected serum samples derived from Afghanistan, Pakistan and Iraq.

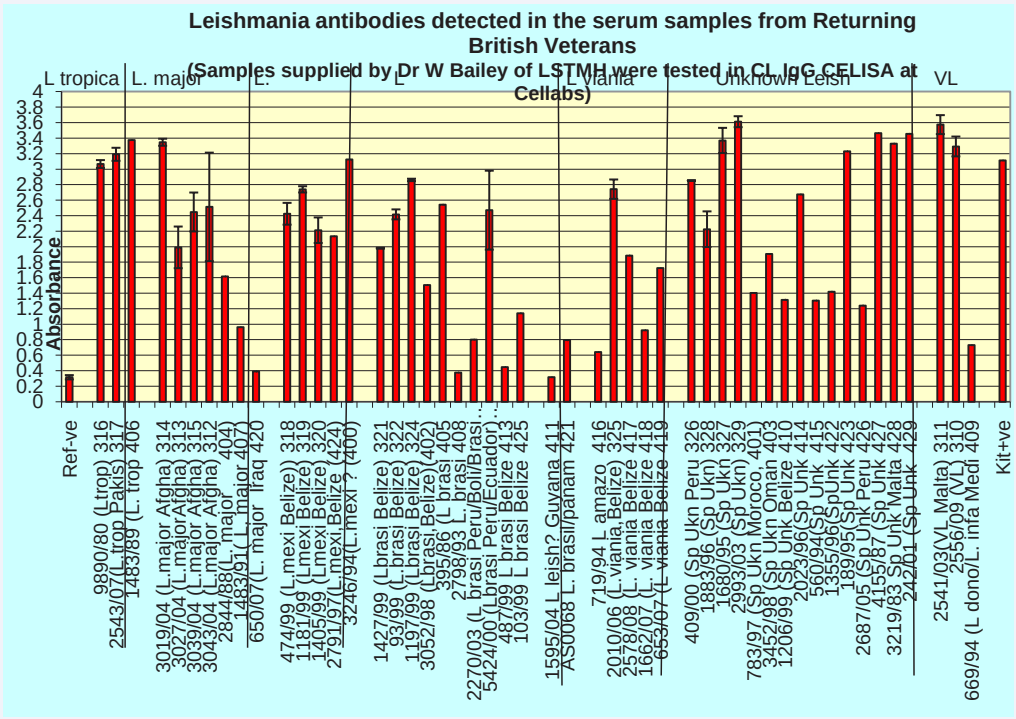

Figure 4 Overall antibody profile of n=50 samples against *L. tropica, L. major, L mexicana, L. brasiliensis, L. viania,* and *L.donovani* (visceral leishmania).

Samples from Central and South American regions

There were 9 samples collectively from Ecuador (WB 323), Peru (WB 323, 326 426), Guyana (WB 411), Brazil (WB405, 408 416), Bolivia (WB 412). Leishmania parasites could not be typed from two samples (WB 326,426), however, these two samples showed antibody response OD >1.0. The remaining 7 species were identified as *L. brasiliensis* (WB 405, 408, 412, 323,411, 421) and *L. amazonensis* ((WB 416). All these samples were positive for antibody.

Samples from Middle Eastern and Mediterranean regions

There were 4 samples from Mediterranean regions (WB 409, 310) Malta (WB311 WB 428), of which 310,311 and 409 were identified as visceral leishmania (*L. donovani/infantum*) parasites. Sample 311 was tested and found to be very strongly positive in VL- kit. This was further titrated up to a dilution of 1/3200, which showed an OD=1.8 indicating very strong antibody levels. Two samples (WB403 from Oman and WB 401 from Morocco) were also tested in CL –Kit and found to be strongly positive (OD >1.4).

Figure (4) represents overview of 50 serum samples tested at the same dilution for level of antibodies. It is clear from this figure that antibody profiles are practically best way to demonstrate the bodily reaction to leishmania infections. Both visceral and cutaneous leishmaniasis shows elevated antibody response.

Additional tests

As the veterans have travelled in different endemic countries, it is quite possible that they were exposed to other infections in those endemic countries. We examined the possibility of malaria exposure with these samples by using two defined and proven ELISA kits. A Pan Malaria antibody ELISA, which is proven to be useful for detecting exposure to any one of four (*P. falciparum, P. vivax, P. malariae and P. ovale*) human malaria species was used to test n=50 samples at 1/100 dilutions. We then tested for the presence of pLDH antigen in the serum samples by testing at 1/20 dilutions. Results are in Table (2). Out of n=50 samples tested, at least n=8 samples showed evidence for malaria exposure with the demonstration of malaria antibodies [n=8 samples were uniformly positive, of which two samples (WB 329 and 429) were very strongly antibody positive]. Only 4 samples were found to be positive for pLDH antigens, suggesting that they were carrying active infection.

DISCUSSION

Most sera samples were derived from British Army Veterans who were deployed into several countries. And also several citizens of Britain visited as tourists. As there were not any prophylactics they couldn't protect themselves against Leishmania parasitic infection. All these visitors irrespective of cadre and position they became vulnerable to infection and they became susceptible subjects. These people on the whole were exposed to a variety of Leishmania species. They formed the basis of this study and the serological assays conducted on their sera sample gave an excellent condition to study humoral response. Antibody response against Leishmania especially that of cutaneous leishmaniasis is poorly reported and many think that it is non existing. We report a high level of antibody response during the clinical conditions caused by the cutaneous leishmania infections. High level of antibody response was predominant during infection with *L. brasiliensis*, *L. mexicana*, *L. viania* in

Table 2: Additional tests (Pan malaria antibody and pLDH antigen detectionELISAs) were performed to demonstrate exposure to malaria.

Sample*	Pan Malaria Antibody CELISA (1/100 dilution)	Pan-LDH ELISA (1/20 dilution)
Ref-ve	0.2 (negative)	0.2 (negative)
L viania Belize WB 417	0.478 (+ve)	0.478 (+ve)
Species Unknown WB329	2.496 (Strongly +ve)	1.487 (+ve)
Sp unknown WB 429	2.966 Strongly +ve)	1.821 (+ve)
L. major Afghanistan WB 314	0.462 (+ve)	0.136 (negative)
Species Unknown (WB327)	0.422 (+ve)	0.21 (negative)
Spp unknown (WB423)	0.507 (+ve)	0.178 (Negative)
VL case (Malta; WB 311)	0.603 (+ve)	0.17 (Negaitve)
VL case (WB 310)	0.46 (+ve)	0.147 (Negative)
Kit+ve	3.299 (+ve)	0.979 (+ve)

*All n=50 samples were tested and only those samples showing clearly higher absorbance values and reagrded as positive are shown below.

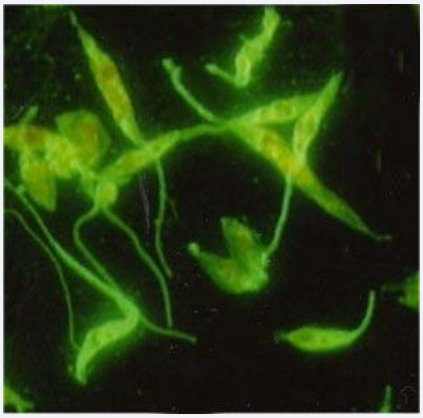

Figure 5 Specific staining of *Leishmania spp* by indirect immunofluorescence antibody test (IFAT). IFAT on promastigote stages.

Belize. We also demonstrate the presence of antibody response in all samples comprising both cutaneous and visceral forms of Leishmania infections. This was made possible by the availability of antibody detection kits from "Good Manufacturing Practice" based specialist commercial company Cellabs Pty Ltd Australia which is a technology recipient by collaboration with the Walter Reed Army Institute of Research, Maryland USA. The ELISA method is based on the usage of cocktail promastigote antigens. The ELISAs were constructed for demonstration of antibodies in visceral leishmania cases by using promastigote exo-antigen from *L. donovani* and *L. infantum*. The ELISAs for the cutaneous form of leishmania infection has utilized exo-antigens of *L. mexicana*, *L. panamensis* and *L. tropica*. This method was found to be reliable due to the fact that all most all samples were antibody positive. Some of the samples showed very high level of antibody response. We collected samples from returning British Army Personnel who visited different countries - Belize, Afghanistan, Pakistan, Iraq and South American countries such as Peru, Ecuador, Bolivia and Guyana and tested for presence of antibodies specific to Leishmania parasites. While appreciating the data presented in this paper, some points need to be emphasized; the samples reflect on the movement of British veterans in different endemic countries where military issue was the main point of duty. How the human body reacts upon exposure to leishmania antigens (e.g. In the form of sand fly bite during combat field operation). Some of the veterans visited several endemic countries and contracted leishmania infection, making it difficult to trace the source of the infection to a particular country it does not reflect on the pattern of infection prevailing in the endemic regions. There are 6 different categories of people in endemic situations that can be seen (a) unexposed. (b) Exposed but not infected, (c) exposed with sub-clinical infection, (d) infected showing clinical symptoms, (e) infected under treatment, (f) treated / cure of infection. Our data unequivocally demonstrated that the veterans were exposed to leishmania infection during the duty-of-care and that the antibody in samples would be a very sensitive indicator for assessing such exposure. Earlier workers could not measure the antibody response, deeming it ineffective [4] and relied upon the other indicators such as parasitology and clinical symptoms. In this study, we have unequivocally shown that the antibody response and also intensity of reactions are effective diagnostics of infection. Recent refugee cases due to civil war in Syria are paramount example of the spread of cutaneous cases. Some 53,000 cases are reported which are showing clinical symptoms. These antibody detection techniques may be of some use in detecting cutaneous cases prior to clinical symptoms. The antibody response is consistently seen in all form of cutaneous leishmaniasis due to *L. brasiliensis, L. mexicana, L. viania*. If one considers the antibody response is vigorous in visceral leishmaniasis, our test results indicate the antibody response with cutaneous sample is equally of high order. In the literature, there has been less emphasis on the antibody response due to any form of cutaneous leishmaniasis. Recently Romero et al.[23], did see antibody response against cutaneous leishmaniasis in Brazil. In our study that notion has been thoroughly changed and the highest antibody response was recorded against cutaneous leishmania parasites.

Large numbers of leishmaniasis cases, of a magnitude not encountered in the United States since World War II have challenged clinicians in both the military and the civilian sectors. In the past years, there were >600 cases of cutaneous leishmaniasis and 4 cases of visceral leishmaniasis diagnosed in American soldiers deployed to Iraq, Kuwait, and Afghanistan [21]. In Operation Iraqi Freedom, US soldiers had intense vector exposures and often reported receiving hundreds of insect bites starting in late April 2003. Over 50,000 sandflies were collected from 14 sites in Iraq, where the infection rate in sandflies was determined by polymerase chain reaction to befrom 0.06 to 2.78%. British soldiers worked alongside US soldiers, meaning they are equally exposed to vector bites and to Leishmania. The antibody responses reported all came from subjects showing clinical symptoms. The detection kits recorded significantly high level of antibody response against cutaneous leishmaniasis. On the whole, we can summarise the value of Exoantigens for detection of antibodies for a variety of Leishmania species.

Large outbreaks of cutaneous leishmaniasis, due to *L.*

brasiliensis and *L. panamensis,* totaled 40,000 cases in Colombia between2005 and2009 in soldiers of the Colombian Army. This outbreak was caused by the influx of military personnel into jungle with the mission of combating illicit crops and guerrilla operatives [14]. Vector-borne diseases have also been shown to emerge during other deployments. During Operation Desert Shield/Storm in eastern Saudi Arabia, 12 cases of viscera tropic and 20 cases of cutaneous leishmaniasis were identified (697,000 allied soldiers deployed cumulative rate of 0.017/1000 and 0.03/1000 cases respectively, as compared to 4.3/1000 cases of cutaneous leishmaniasis seen in Colombian Army) [15]. During 2005 -2012, the disease was contracted by 195 patients in the Netherlands, most of them from military [16]. Cutaneous leishmaniasis is an increasing problem in British military and naval personnel deployed in Belize, Iraq and Afghanistan [17,18]. Based on clinical symptoms in British military personnel, the disease has been diagnosed; yet immunological reactions have not been studied. This is first time the samples from these personnel is subjected to any immunological testing by which to characterize the antibody profiles. Exoantigens of promastigote stages are useful in demonstrating specific antibody response in individuals suffering from leishmania infection. No study has been done on the seroconversion with leishmania parasites. However, during 2008-2011, using serum samples from 467 UK military personnel, they reported 3.1% seroconversion rate to Rickettsia, Coxiella burnetti, sandfly fever virus, and hanta virus. Seroconversion was not observed for infections by Crimean-Congo hemorrhagic fever virus [19]. Our study clearly showed the antibody response to cutaneous and visceral leishmania infection. These samples are derived from the clinical cases and they were tested in blind. Antibody detection ELISA is considered to be highly sensitivity compared to indirect fluorescence antibody test and polymerase chain reaction [20]. There was incidence of malaria, which can be detected by specific immunological testing. Such a response against Leishmania and Malaria is ascribed due to A2 gene product which is reported previously [22].

CONCLUSION

Specific antibody responses to *Leishmania* parasites has been reported here with clinical samples collected from military personnel visiting Belize, Afghanistan, Iraq and other endemic areas. Although many reports of leishmania cases based on clinical symptoms are existing from military personnel, the specific antibody profiles from these cases were not reported earlier. The solid phase immunoassay becomes an easier tool to use to investigate the circulating antibody against the infection. These are available for diagnosis of visceral form, as well as various forms of cutaneous leishmania infections. In that way, antibody response is the earliest response seen in any personnel visiting potential endemic areas as an indication of the exposure of infection. Antibody profiling showed that samples collected from different areas proved to be positive demonstrating that the infection is detectable by serological analysis. It is shown that antibody detection is very sensitive and early detection is possible, allowing for accurate and expedient identification of individuals needing treatment.

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