Global Advanced Research Journal of Medicine and Medical Science (ISSN: 2315-5159) Vol. 4(2) pp. 078-082, February, 2015
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Full Length Research Paper

Sensitivity of aqueous and freeze-dried DAT (AQ-DAT and FD-DAT) in diagnosis of visceral leishmaniasis in Sudan- Possibility of adapting the DAT (aqueous antigen) as a rapid test by using blood spot on filter paper

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Accepted 09 February, 2015

Leishmaniasis is the second largest parasitic killer in the world. In developing countries with large numbers of patients in rural areas, simple diagnostic tools are necessary for field use. Our study aimed to compare the performance of the aqueous with freeze-dried DAT (AQ-DAT and FD-DAT) in diagnosis of visceral leishmaniasis and to investigate the possibility of adapting the DAT (aqueous antigen) as a rapid test by using different sampling method (blood spot on filter paper and serum from venous blood) with different elution times and different incubation times, and to determine the significant titre in each method. The sensitivity of FD-DAT and AQ-DAT at a cut-off titre 1:3200 was found to be 100% as both antigens gave a significant diagnostic titre for all of the parasitologically confirmed VL cases. We concluded that AQ-DAT and FD-DAT have a very high sensitivity for the diagnosis of visceral leishmaniasis at a cutoff titre of 1:3200, with a fair agreement between the two tests (Kappa measure of agreement =0.21). We also noticed that using filter papers have excellent results especially when taking an elution time of eighteen hours.

Key words: Visceral leishmaniasis (VL), aqueous DAT (AQ-DAT), freeze-dried (FD-DAT).

INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites of the Leishmania genus. This disease is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 200,000 to 400,000 infections each year worldwide (World Health Organization, 2014; Desjeux, 2001).

Visceral leishmaniasis (VL) is a chronic parasitic disease caused by the *Leishmania donovani* complex in

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East Africa and the Indian subcontinent and by *Leishmania infantum* (syn. *Leishmania chagasi*) in Europe, North Africa and Latin America. VL is fatal if left untreated and *L. guayanensis* is transmitted in one of two ways. Zoonotic VL is transmitted from animal to vector to human, and anthroponotic VL is transmitted from human to vector to human. In the former, humans are occasional hosts and dogs are the main reservoir for *Leishmania*, particularly in rural and urban domestic environments (Chappuis et al., 2007; Regina-Silva et al., 2014).

Leishmaniasis occurs in three clinical forms: (i) cutaneous leishmaniasis (CL), which is caused by *L.major*, *L. tropica*, *L. aethiopica* (Old World CL), L.infantun, L. chagasi (Mediterranean and Caspian Sea region CL), *L. amazonensis*, *L. mexicana*, *L. braziliensis*, *L. panamesis*, and *L. peruviana* (New World CL); (ii) mucocutaneous leishmaniasis (MCL) or espundia, which is caused by L. brazilensis, *L. panamensis*, *L. guyanensis* in the New World and is occasionally encountered in the Old World, caused by *L. infantum* and *L. donovani*; (iii) visceral leishmaniasis (VL), which is caused by species of the L. donovani complex that consist mainly of *L. infantum*, *L. donovani*, and *L. chagasi*. LV is also known as kala-azar, black fever, and Dumdum fever. There is a fourth form, known as diffuse cutaneous leishmaniasis (DCL), which is caused by *L. amazonensis* and *L. aethiopica* (Singh, 2006; Elmahallawy et al., 2014).

In developing countries where the disease is not prevalent, the existence of laboratory facilities enables an adequate and efficient follow-up of the disease. However, in developing countries with large numbers of patients in rural areas, simple diagnostic tools are necessary for field use (Santarém and Cordeiro, 2007). Laboratory diagnosis of VL includes microscopic observation and culture from adequate samples, antigen detection, serological tests, and detection of parasite DNA (Elmahallawy et al., 2014). The direct agglutination test (DAT) is based on direct agglutination of Leishmania promastigotes that react specifically with anti-Leishmania antibodies in the serum specimen, resulting in agglutination of the promastigotes. Whole, trypsinized, coomassie-stained promastigotes can be used either as a suspension or in freeze-dried form that can be stored at room temperature for at least two years, facilitating its use in the field (Fakhar et al., 2012).

This study aimed to compare the performance of the Aqueous with freeze-dried DAT (AQ-DAT and FD-DAT) in diagnosis of visceral leishmaniasis and to investigate the possibility of adapting the DAT (aqueous antigen) as a rapid test by using different sampling method (blood spot on filter paper and serum from venous blood) with different elution times and different incubation times, and to determine the significant titre in each method.

MATERIAL AND METHODS

A descriptive comparative hospital-based study was carried out to evaluate the sensitivity of the DAT test using the locally produced aqueous antigen, compared to that of the dried antigen. Patients were collected from Tropical Teaching Hospital and Khartoum Teaching Hospital (Sudan). Twenty one patients with confirmed visceral leishmanisis (VL), based on parasitological diagnosis through bone marrow or lymph node aspiration, were included in the study. A pre-coded questionnaire was designed to collect the personal, epidemiological and clinical data from individuals included in the study. Individuals were sorted out based on the clinical and parasitological manifestations. The criteria for clinical selection of VL suspect were based on the presence of fever for two weeks or more, splenomegaly and / or lymphadenopathy and exclusion of malaria. Then the clinical history, demographic data and clinical examination were filled in the precoded questionnaire.

Blood protein eluates were prepared from the filter papers in V-shaped microplates. From each blood spot, a 5 mm disc was punched out and placed in a well of the microplate. Then, 125 µl of buffer was pipette into each well. The plate was covered to avoid evaporation and incubated at 4°C. Assuming a mean haematocrit value of 50%, a 5 mm disc of blood-impregnated filter paper contains approximately 5 µl blood or 2.5 µl serum. The eluate in 125 µl of DAT buffer thus corresponds to a serum dilution of 1: 50. From the blood eluates, serial twofold dilutions of 1: 100 ul to 1: 12800 were then made in a freshly prepared diluent. Eluates were collected at two different elution times (after 8 and 18 hours), using two different buffers (normal saline alone and normal saline with 2 mercaptoethanol).

The DAT was performed essentially as described by Elharith *et al* (1986). Briefly, serum samples were diluted in a dilution solution containing 0.9% (wt/vol) NaCl, and 0.78% (vol/vol) β-mercaptoethanol. To discriminate between agglutination and nonagglutination, the use of V-shaped microwell plates (Greiner, Frickenhausen, Germany) was mandatory. A twofold dilution series of the sera was made, starting at a dilution of 1:100 (step 1) and going up to a maximum dilution of 1:25600. Prior to its use, aliquot of FD antigen was reconstituted in 5 ml of normal saline (0.9% [wt/vol] NaCl). Reconstituted antigen (50 µl) was added to each well of the microwell plate containing 50 µl of diluted serum. The titer was defined as the highest dilution at which agglutination was still visible. In comparison to the blue dots present in the negative control wells, this agglutination showed as blue mats, enlarged blue dots with frayed edges, or enlarged blue dots.

Table 1. Sensitivity of FD and AQ-DAT at a Cut-off titre of 1:3200

		Freeze Dried DAT		Aqueous DAT	
		Positive	**Negative**	**Positive**	**Negative**
Bone marrow aspirate	**Positive**	21/21	0/21	21/21	0/21
	Negative	0/21	0/21	0/21	0/21
Sensitivity		100%		100%	

Table 2. Performance of FD and AQ-DAT at a Cut-off Titre of 1:6400 in 21 parasitologically confirmed patients with visceral leishmaniasis:

		Freeze Dried DAT		Aqueous DAT	
		Positive	**Negative**	**Positive**	**Negative**
Bone marrow aspirate	**Positive**	19/21	2/21	18/21	3/21
	Negative	0/21	0/21	0/21	0/21
Sensitivity		90.5%		85.7%	
95% CI		71.1 to 97.3		65.4 to 95	

Table 3. Agreement analysis between the two tests AQ-DAT and FD-DAT:

		Aqueous DAT Antigen			Total
		3200	**6400**	**12800**	
	3200	**0**	**1**	**1**	**2**
Freeze Dried DAT Antigen	**6400**	**1**	**1**	**1**	**3**
	12800	**2**	**1**	**13**	**16**
Total		**3**	**3**	**15**	**21**

The AQ-DAT was performed in a similar way as the FD-DAT mentioned above, but the diluent solution used contained 0.2gm gelatin for every 25 ml of the solution containing 0.9% NaCl and 0.78% β mercaptoethanol.

The sensitivity of the DAT (AQ) and DAT (FD) were calculated according to the following formula:

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False Negative}}$$

An excel sheet was designed to summarize the results of laboratory testing in the field. Data from the questionnaire and excel sheet were entered and analyzed by computer using Special Package for Social Sciences (SPSS) software. Simple descriptive statistics, frequency distribution and cross tabulations were obtained. Significance test was performed to compare the results among various groups in the study population. Kappa measure of agreement was calculated. The interpretation of the kappa values was performed according to the criteria of Landis and Koch ,who recommended that a value greater than 0.8 be considered 'almost perfect', between 0.6 and 0.8 'substantial', between 0.4 and 0.6 'moderate', between 0.2 and 0.4 'fair', between 0 and 0.2 'slight', and between 0 and -1 'poor' (Landis and Koch, 1977).

RESULTS

The sensitivity of FD-DAT and that of AQ-DAT at a cut-off titre 1:3200 are shown in table 1. Both antigens gave a significant diagnostic titre for all of the parasitologically confirmed VL cases. The sensitivity of FD-DAT using blood eluted at 8 hours was 80%. The sensitivity of AQ-DAT was found to be 85.7% when the titre of significance was regarded as 1:6400 as shown in table 2. Titres obtained by FD and AQ-DAT at Cut-off: 1:3200, 1:6400 and 1:12800 in the 21 parasitologically confirmed patients with visceral leishmaniasis are shown in figure 1.

Agreement analysis between the two tests; AQ-DAT and FD-DAT using Kappa measure of agreement (Kappa measure of agreement = 0.21), indicated a fair agreement between the two tests (table 3).

McNemar test showed no significant difference (p =0.5) between FD-DAT performed on filter paper blood eluates in Normal Saline at 8 and 18 hours at a cutoff titre of 1:3200. Comparison between FD-DAT performed on filter paper blood eluates in DAT diluent different at 8 and 18 hours at a cutoff titre of 1:3200 revealed no significant difference (p = 0.06) as indicated by McNemar test.

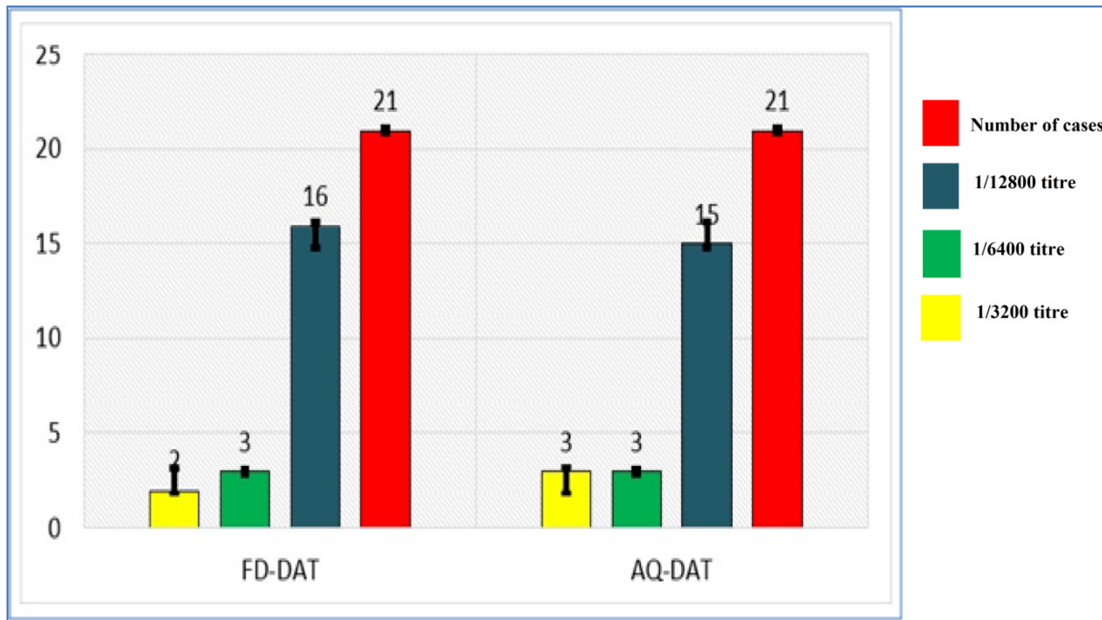

Figure 1. Titres obtained by FD-DAT and AQ-DAT at Cut-off: 1:3200, 1:6400 and 1:12800 in 21 parasitologically confirmed patients with visceral leishmaniasis.

DISCUSSION

The most important reported results that have emerged from some previous studies were that high ambient temperatures and lack of cold chain facilities in most of the VL-endemic areas have limited application of DAT despite the fact that the technique is simple and applicable (Mohebali et al., 2011; World Health Organization, 2010).

From our results it was found that DAT test using aqueous and freeze dried antigens gave an identical sensitivity of 100% at a cutoff titre of 1:3200. At a cutoff of 1:6400 it was found that the sensitivity of AQ-DAT and that of FD-DAT was 90.5% and 85.7% respectively. This is in concordance with some recent study carried out by Kakooei et al (2014) who indicated that there were no significant differences between freeze-dried and non-freeze-dried sera over a period of 11 months at certain temperatures (Kakooei et al., 2014).

Our study also showed a fair agreement between the two tests (kappa value was 0.21). This is in agreement with that done by Jacquet et al (2006) who found high agreement between results obtained with LQ DAT and FD DAT (Jacquet et al., 2006).

A similar result of the sensitivity and specificity was obtained in a study conducted in india (Mandal et al., 2008). But many other studies showed lower values of both sensitivity and specificity. Another study conducted in India showed that the sensitivity of FD-DAT and AQ-DAT of 96% (95% CI 91—98) and 97% (95% CI 93—99) respectively, and specificity of 93% (95% CI 86—97) and 94% (95% CI 87—98) respectively (Sundar et al., 2006). Many studies in Sudan concluded that the DAT was highly sensitive (94-100%) and specific (74-100%) (Osman et al., 2000).

From our study we concluded that AQ-DAT and FD-DAT have a very high sensitivity for the diagnosis of visceral leishmaniasis at a cutoff titre of 1:3200, with a fair agreement between the two tests (Kappa measure of agreement =0.21). We also noticed that using filter papers (FD-DAT performed on filter paper blood eluates in Normal Saline) have excellent results especially when taking an elution time of eighteen hours. These may be promising results for using of filter papers instead of serum in the areas with poor facilities, although further studies on using of filter papers are needed because of the limited number of cases included in this study.

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