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Detection of anti- leishmanin antibodies by direct agglutination test using freeze dried antigen in Basrah province

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Abstract

The prevalence of anti-leishmanin antibodies in (253) human serum samples, were tested by direct agglutination test (DAT) which using developed antigen based on freeze-dried antigen. It was shown that freeze dried antigen active, even storage at ambient temperature for several months. The test shown that the cut-off titre of 1:800, 181 (90.9%) of 199 serum samples from confirmed visceral leishmaniasis patients tested positive. The specificity of test was 100%.

Introduction

Visceral leishmaniasis (VL) caused by intracellular protozoan parasites of the genus *Leishmania*, is a zoonotic disease whose main reservoir are dogs. Leishmaniasis is a serious public health problem in under developing areas of the world, is a potentially fatal disease that affect an estimated 500,000 people each year (WHO,1997).

The routine diagnosis of visceral leihmaniasis may be based on one or more of the following methods (i) the microscopical detection of the parasite in smears of bone marrow or lymph node (ii) the culturing of parasite from patient material or (iii) serological test for the detection of anti- *Leishmania* antibodies. A relatively new and very sensitive technique for the detection of *Leishmania* parasite in blood is PCR and dipstick (Piarroux, *et al*. 1994;). Compared with the first two diagnosis mention above, serological tests have the advantage that blood sampling

is relatively easy, with little inconvenience for the patient, and that many samples may be processed simultaneously (Meredith, *et al*. 1995).

The direct agglutination test (DAT) remains the first line diagnostic tool for visceral leishmaniasis in many developing countries. The DAT is a relatively simple test with high sensitivity and specificity that uses whole stained suspension promastigote (Harith, *et al*. 1989).

Human visceral leishmaniasis is a severe health problem in many countries a round the world. Hence, a cheap, reliable and accurate diagnostic test is required to fight this disease which in this study is undertaken by developing the DAT based on freeze dried antigen for the detection the antibodies of this disease.

Materials and Methods

Sample source:

The strain of the *Leishmania donovani* culture MHOM /IQ /1982 / BRCI/AA3 brought from Leishmania unit/ Al Nahrain university. This strain was cultured serially on the diphasic medium (Meredith, *et. Al*. 1995).

Serum samples:

A total of (253) serum specimens were collected from Basrah hospitals in 2006/2007. The specimens divided into three different groups of subject:

(i) There are (199) patients with parasitologically proven. All patients had clinical signs of visceral leishmaniasis.

(ii) There are (15) healthy controls. They had no history of visceral leishmaniasis and no clinical symptoms of the disease.

(iii) There are (39) patients with a variety of disease other than visceral leishmaniasis, there are Brucellosis (n=4), Toxoplasmosis (n-8), Hydatidosis (n=8), Intestinal amoebiasis (n=7), Giardiasis (n=5), and Typhoid (n=7).

Preparation of antigen:

Aqueous antigen was prepared essentially as described by (WHO, 1996).

After harvesting, the promastigotes were washed with Locke solution and treated with 1.2% mercaptoethanol at 37˚C for 45 minutes, the pellets

were resuspended in cold Locks solution to conc. of approximately 2×10^8 (for freeze dried antigen), after which the promastigotes were fixed by treatment with 2% formaldehyde in Locks solution for 20 hours at 4˚C. following washing in cold saline sodium citrate, the fixed promastigotes were stained for 90 minutes with saline-citrate solution containing 0.02% Coomassie birilliant blue. Subsequently the stained promastigotes were washed with the saline citrate solution. Part of antigen was put into freeze-drying solution and 5ml aliquots were freeze dried and sealed. This freeze dried antigen forms the basis of diagnostic for detection of anti-leishmanian antibodies.

Performance of the DAT:

The DAT was performed essentially as describe by (WHO, 1996). Briefly, serum samples were diluted in 1/50 (20µl serum + 980 µ diluents). The use of V-shaped microwell plates, for 12 row microtitre, (Twofold dilution series of the sera was made) pipetted 50 µl of dilution to all wells, into well 2 pipette 50µl of 1/50 dilution of serum, hence well No.1 used as a negative control. 50µl from well 2 to well 3 was transferred and mixed, then 50µl transferred from well 3 to well 4, this operation was continued a cross the plat to well 12.

Positive and negative controls should be systematically incorporated in separated well. Prior to its uses, aliquots of freeze-dried antigen were reconstituted in 5ml of normal saline (0.9% NaCl). Reconstituted antigen 50µl was added to each well of the microwell plate containing 50µl of diluted serum. The plate then covered with plastic film and incubated at 3-6 hours at room temperature. Microtitre plate was placed on a plain sheet of white paper on a light box and viewed the plat from above. The end point was estimated by locating a clear sharp edged, which agglutination is still visible in comparison to the blue spot present in the negative control well. This agglutination shows as blue mats, enlarged blue dots with fraged edges or enlarged blue dots.

Stability of antigen:

Freeze dried antigen was stored at room temperature. After various of time the antigen was tested for its activity.

Results

Stability of antigen:

After storage the antigen at room temperature for several months (10 months), the freeze dried antigen remained fully active in the DAT. Statistically analysis showed no systematic month to month variation in the test.

Sensitivity and specificity:

The sensitivity of DAT is defined as the percentage of serum samples from group one with titre equal to or higher than the cut-off titre. Results are shown that 181 of serum samples from VL patients gave titre $\geq$ 1:800. The titre of 1: 800 was taken as the cut-off point as non of healthy and disease control sera did the titre exceed 1:400. this results representing a sensitivity of 90.9% in freeze dried antigen compared with 89.4% with aqueous antigen.

The specificity of the DAT is defined as the percentage of serum samples from groups (2 and 3) with a titer lower than the cut-off titre 1:800. Results of the present study shown there is no cross reactivity of serum samples from patients suffering from different disease than VL and the other healthy control. Therefore, the specificity of DAT was 100% in using the freeze dried and aqueous antigen.

All the 253 samples described in the materials and methods section were tested in the DAT as describe above. Results for the various groups are given in Table, (1).

Table, (1): The results of various groups obtained in the DAT test by using freeze dried antigen

Titre	Aqueous antigen			Freeze dried antigen		
	Group (i)	Group (ii)	Group (iii)	Group (i)	Group (ii)	Group (iii)
1:100	10	11	36	9	11	37
1:200	8	4	4	7	4	3
1:400	3	-	1	2	-	1
1:800	15	-	-	14	-	-
1:1600	8	-	-	9	-	-
1:3200	18	-	-	17	-	-
1:6400	23	-	-	23	-	-
1:12800	29	-	-	29	-	-
1:25600	29	-	-	28	-	-
1:51200	28	-	-	27	-	-
1:102400	34	-	-	34	-	-
Total	199	15	41	199	15	41
sensitivity	89.4%			90.9%		

Group (i): Confirmed VL., Group (ii): Healthy control, Group (iii): other disease

Discussion

The DAT for detection of anti-*Leishmania* antibodies in serum samples is simple to perform and does not require specialized equipments. The cut-off titre was previously determined 1:800 with aqueous antigen (Jassim, 1998; Mehdi, 2004), and 1:3200 by (Harith, *et al*. 1988). In all these studies the antigen was made from *Leishmania donovani* promastigotes and needed for 18 hours to give the results. So far, they found the testing with aqueous antigen remained stable at 20˚C for less than three weeks and then the reading of the test became impossible (Gari-Toussaint, *et al*. 1994; Meredith, *et al.* 1995). Hence,

when no cooling facilities are available, as is often the cases in the areas where VL is most frequently encountered.
In contrast, the present study shown the development of freeze dried antigen remained fully stable for 10 months at 37˚C, and faster to detection of anti-Leishmania antibodies in the serum samples than the aqueous antigen by giving the result with no more than three hours. This result agree with Oskam, *et al*. 1999; Schoone, *et al*. 2001, which they found the freeze dried antigen is full stable often storage for 18 months at 56˚C. These results indicated that the use of freeze dried antigen in the DAT is feasible.

The present study shown that the performance of freeze dried and aqueous antigen in the DAT when compared, find that the cut-off titre was same with had the same results. While, other studies found the test by freeze dried antigen have a titre that almost one dilution step lower than the titre obtained with aqueous antigen (Meredith, *et al*. 1995). Also, for concerned the value of cut-off titre that presented in the study, 41 serum samples from healthy control had no history of VL were tested. The results clearly indicated that no samples exceeded the titre 1:800.

In the present study fined the sensitivity of the freeze dried antigen were 90.9% with no statistically difference with the aqueous antigen 89.4% when using the cut-off titer 1:800. the choice of cut-off titer depending on the particular wishes with regard to sensitivity and specificity. This result agreement with (Zijlstra *et al*. 1991), in contrast, the sensitivity was found 100% by (Abdel Hameed, *et al*. 1989; Hailu, 1990). The difference in results of DAT in sensitivity was depending on the cut-off titre. The lower of sensitivity in the present study compared with highest one may be due to the lower titre and to the presence of samples from patients with recent infection, from which is known that antibody level may be low (Shiddo, *et al*. 1995).

The specificity of DAT using freeze dried antigen was 100%. No cross reactivity was observed with serum samples from patients suffering from other disease. This results agree with Scott, *et al*. 1991; Meredith, *et al*. 1995.

So, this laboratory results in present study led to investigate the performance of the DAT with freeze dried antigen in a field because it had stable in ambient temperature and it can storage it for longer time than aqueous antigen that may be deterioration due to the suboptimal cooling in the field. Moreover, it show no statistically significant difference between the aqueous and freeze dried antigen was observed in the value of titre.

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تحديد الاجسام المضادة لليشمانيا باستعمال تجفيد الانتجين بطريقة التلازن المباشر في محافظة البصرة

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الخلاصة

تم تحديد الاجسام المضادة لليشمانيا بواسطة فحص مصل (253) شخص باستعمال طريقة التلازن المباشر (DAT) بتطوير تجفيد الانتجين السائل، فقد لوحظ فعالية الانتجين لعدة اشهر رغم حفظه في درجات الحرارة الاعتيادية. حيث وجد ان حساسية الاختبار عند نقطة القطع 1:800 (90،9%) وذات خصوصية 100% للاشخاص المثبتة اصابتهم باليشمانيا.