Evaluation of Dot. ELISA test for Diagnosis Visceral Leishmaniasis in Infected Children

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Abstract

For the first time in Iraq, this study was conducted to evaluate the usefulness of Dot.ELISA, for detecting anti - *Leishmania donovani* antibodies in serum samples from suspected patient (children under 8 years) with Visceral Leishmaniasis V.L.. Sera from 73 V.L. , 60 Healthy controls, and 57 patient with other parasitic diseases other than V.L. (Amoebiasis, Giardiasis , Toxoplasmosis, Schistosomiasis , Hydatidosis, Ascariasis , Lupus Erythromatosus , Viral Hepatitis, and Cutaneous Leishmaniasis) were examined. Anti *Leishmania donovani* antibodies detected in 71 out of 73 suspected Visceral Leishmaniasis . Data of this study showed that infection in male group was more than female group. Result of this study revealed a high prevalence of patients with V.L. infection at the age range 1 - 2.9 and 3 - 4.9 years when compared with other range groups ($P>0.01$). A cross – reaction was found with sera Lupus Erythromatosus, No cross - reaction was found with sera from other disease . Sensitivity was determined as 97.3 %, and Specificity was found to be 95.7% . Positive and Negative the Values Predictive of test were 96% and 97.4% Dot .ELISA is simpler, Serodiagnosis Rapid, sensitive and specific, Its use in respectively. Seeing that of V.L. can be recommended.

Introduction

Visceral Leishmaniasis V.L. the second largest parasite killer in the world (after malaria)which is responsible for an estimated 500,000 new cases of V.L. occur in each year and a tenth of these patients would die [1]. In Iraq especially V.L. was regarded as an endemic disease[2].Demonstration of parasites in bone marrow aspirates or needle biopsy specimens of the spleen, and lymph node, or by *vitro* cultivation are the definitive methods of diagnosis [3]. However, these methods are insufficiently sensitive, and the techniques are invasive, painful , and even hazardous [4]. A number of serological tests have been developed and evaluated for the diagnosis of V.L., DAT [5] and ELISA [6, 7]. Later flow using recombinant antigen [7,8] and PCR [9].In Iraq, various epidemiological survey of V.L. was done by using IFAT technique [10]. This test requires more expensive and specialized equipments and it is not suitable for large scale examination of sera [11]. Various types of ELISA has been developed and described for competition , the direct method for detection antibody[6] and the double antibody sandwich method for detection antigen [7]. It is usful in field owimg to its simplicity Dot .ELISA has been succefully applied to the Serodiagnosis of parasitic diseases, because it is simple to perform, inexpensive , and highly accurate and serum conservative [5]. The present study aimed to evaluate the Dot. ELISA for Serodiagnosis of V.L. by using Promastigote antigen and human sera infected with V.L.

Material and Methods

Venous blood samples were collected from 73 Children under Eight years suspected to have V.L. from Child's Central Teaching Hospital in Baghdad during April- September 2007. V.L. was diagnosed on the basic *Leishmania serology* (IFATor Dipstck) . *leishman donovan* bodies were detected bone marrow aspirates . Control serum samples were obtained from 6o Health subjects with no known exposure to V.L. . Cross reactivity were performed by using 57 samples from patient with parasitic infection than V.L. , Including Amoebiasis (n=5) , Giardiasis (n=5) , Toxoplasmosis (n=5) ,Schistosomiasis (n=5), Hydatidosis (n=5), Ascariasis (n=5) , Viral Hepatitis (A=5/B=2) , Lupus Erythromatosus (n=5), and Cutaneous Leishmaniasis (n=15).

Preparation of Antigen

The primary isolation of parasites from bone marrow aspirates of suspected V.L. patients were grown in semisolid medium which prepared according Limoncou *etal.,* [12]. Some of the aspirates were inoculated in Novey, Mac Neal, Nicol (NNN) medium at 25°C to isolate, and culture visceral stock [9]. After the Promastigotes of *Leishmania donovani* growing with 7-10 days [13] on Semisolid medium were transferred to 100ml sterile containers, and then 10ml of liquid medium was added [14]. The container was incubated at 25°C in orbital incubator. The growth of parasites were examined after two days to ensure a good growth and sterilely. Other quantities of liquid medium were added when needed. The parasites were amplified on liquid medium after 3-5 days later, a drop of culture were examined to check the presence of , sufficient number of Amastigote . The culture characteristically contains ≥97% motile Promastigotes. The Promastigotes were collected by centrifuging the liquid medium overlay at 750g for 15 min at 4 °C. The pellets were washed three times with PBS, parasites fixed in 50ml of 1.5% formalin - PBS fore 1hr. at 22°C and washed three times in Triethanol amine Buffered Saline(TBS),pH7.5, and estimation of the protein content of antigen to the method of Bradford [15].

Dot.ELISA procedure:

Dot. ELISA was performed as described by Pappas *etal.,* [16]. A nitrocellulose filter discs (25 mm diameter,0.22µl pore size, Millipor,Inc., Bedford.MA) cut into 5 x5mm square, and all discs are handled with forceps to prevent contamination. 1 µl Promastigotes antigen (4.2 x 10^5 Promastigotes, 1 µg protein) was dotted onto each disc by10 µl Hamilton syringe . Antigen was stabilized on the discs by drying for 10min.at 56°C. Antigen discs were then stored at -20°C until used [16]. All steps were preformed at room temperature 22-24 °C an all working solution were made in TBS. Before antigen discs were left in room temperature, then placed in wells of flat-bottom 96-well micro liter plates (Maxi Sorp., Nune, Roskilde, Denmark).

Un bound sites on the discs were blocked with 75 µl of 5% of Bovine serum albumin BSA – TBS (w/v).The micro titer plates were shaken fore one minute, then incubated for 15 min. The procedure adequately blocked all nonspecific protein binding sites on antigen discs and all surfaces. Blocking solution was aspirated off the antigen discs and 50 µl of 4 fold dilution of saea in 1% BSA -TBS (w/v)was added to each well. Plates shaken for 1min. and incubated for 30min. Sera were aspirated off and washed three times in 100 µl of 0.05 Nonided P-40 (NP-40.PH6.85,was purcosed from Sheel Chemical Co., Ltd. West Orange, NJ). During the third wash antigen discs were incubated in NP-40-TBS for 10 min. before aspiration 50 µl hors radish peroxidase (HRP)conjugated goat antihuman IgG (Sigma Chemical) diluted 1:100 in 1% BSA – TBS (pH7.4) was pipette in 50 µl volumes into each well and the plates were shaken then incubated for 30min. conjugated aspirated off all test samples and the plates were washed as described above. Freshly prepared substrate by mixing together 2ml of 4- Chloro – 1- naphthol

4CIN, a pricipitable substrate , was dissolved in anhydrous methanol (3 mg / ml) and stored in a brown bottle in the dark at 4°C . Every 14 days this stock solution was prepared fresh. Immediately to be for use, 10ml of TBS and 4 µl of 30% H_20_2 were added to 2 ml of 4CIN. Fifty micro liters of this solution (PH 7.35 - 7.4) were added to each test well and the plates were shaken and incubated for 30min. Substrate was aspirated and the plates were washed three times in TBS and were allowed to dry. Serum dilutions causing the development of well- defined blue dots on antigen discs were considered positive.

Statistical analysis: All the statistical analysis were done by zar [17].

Results

Table (1) showed that 71 out of 73samples from suspected V.L. male patients 46 , 64.8 were higher than number and percentage of female patients 25, 35.2 %. Data demonstrated in Figure(2) showed that the different prevalence of V.L. in patients with represent to age groups range 1-2.9 and 3-4.9 years (46.57% ,n=33) and (26.8%, n=19) were respectively more prevalence of significance was (P>0.05). For Dot .ELISA , 71 sera from suspected patients, blue dot were observed against 4 fold dilutions of sera. All the control sera (60 cases) gave negative results. A cross – reaction was observed with serum samples from patients with Lupus Erythromatosus .Whereas as serum samples from patients with (Amoebiasis , Giardiasis, Toxoplasmosis ,Schistosomiasis , Hydatidosis , Ascariasis , Viral Hepatitis , and Cutaneous Leishmaniasis) didn't cross react.Sensitive of 97.3%(71 positive of 73 V.L. patient samples), and the specificity was found to be 95.7% (112 negatives of 117 non patients samples).Considering the above findings, Therefore positive and negative predictive values of the assay were 96%,97.4% respectively.

Discussion

Numerous epidemiological and clinical studies had noted difference in the prevalence of *Leishmania* disease between males and females in Iraq[10], Al-jeboori [18] found that male : females was 3:1 in the part central part of Iraq. There were numerous observations which demonstrated that the difference in the prevalence of V.L. disease between males and females was related to the difference in the host immune response to infection played an important role in the resistance and susceptibility of infection due to increased expression of INF -gamma in females in comparison with males which led to increased female protection against the infection [19]. V.L. occurs mostly in young population. The results showed that the prevalence of the disease described in patients with increasing of age. Similar results were obtained in other studied in Iraq and neighboring countries [10, 20, 21, and 22]. These findings might be due to difference in the host immune response (humoral and cell mediated immune response), that will be considered infant is not well developed at an early stage of life. The higher rate at age of 1-2.9 years might be due to the difference in susceptibility of population to the infection. Another possible cause for the infection of the infants was that the sand flies which have better opportunities of biting relatively immobile infant than other children [23]. The Dot. ELISA has been successfully applied in Serodiagnosis of many parasitic diseases and has several advantages [24]. The results can be read with naked eye and the antigen dotted nitrocellulose membrane strips can be stored for 3 years at 4°C. As demonstrated in Yamaura and Arak study [25], it has been also reported that antigen – dotted nitrocellulose membrane stored for up to 3 months at room temperature 37°C.

The sensitivity of the Dot. ELISA in the present study 97.3 % and specificity was 95.7 % in the diagnosis of V.L. positive negative predictive values were 96 % and 97.4 % respectively. Our data agreed with Dietze study [26] which improved that Dot. ELISA test with high

sensitivity of 97% and specificity of 100 % in diagnosis of Canine V.L. Our finding is consist with Ali study[24] in Iraq , which found that sensitive 97.3% and specific 95.7% for the diagnosis of human Hydatidosis. Hatam *etal*., [27] used Dot. ELISA to Serodiagnosis of Amoeboasis was found to be more specific 96.3% and sensitivity 83.3 % , Positive and Negative Predicative values were 93.7% and 89.6% respectively. Cross- reactions were observed between test antigen and sera from 5 patients with Lupus Erythromatosus. This was most likely due to lupus patients anti – nuclear antibodies reaction with parasite DNA. These cross reactions were however, not high cross- reactivity with sera from patients with confirmed

Cutaneous Leishmaniasis was not observed .This is most likely due to the observation that serum antibody levels are characteristically lower in cases of Cutaneous Leishmaniasis [16]. Dot. ELISA had a higher specificity and sensitivity and since it is a simple , no expensive and reliable test , its use can be recommended.

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Table (1) Distribution of V.L. patients according to gender

Gender	Patients No.	%
Male	46	64.8
Female	25	35.2
Total	71	100

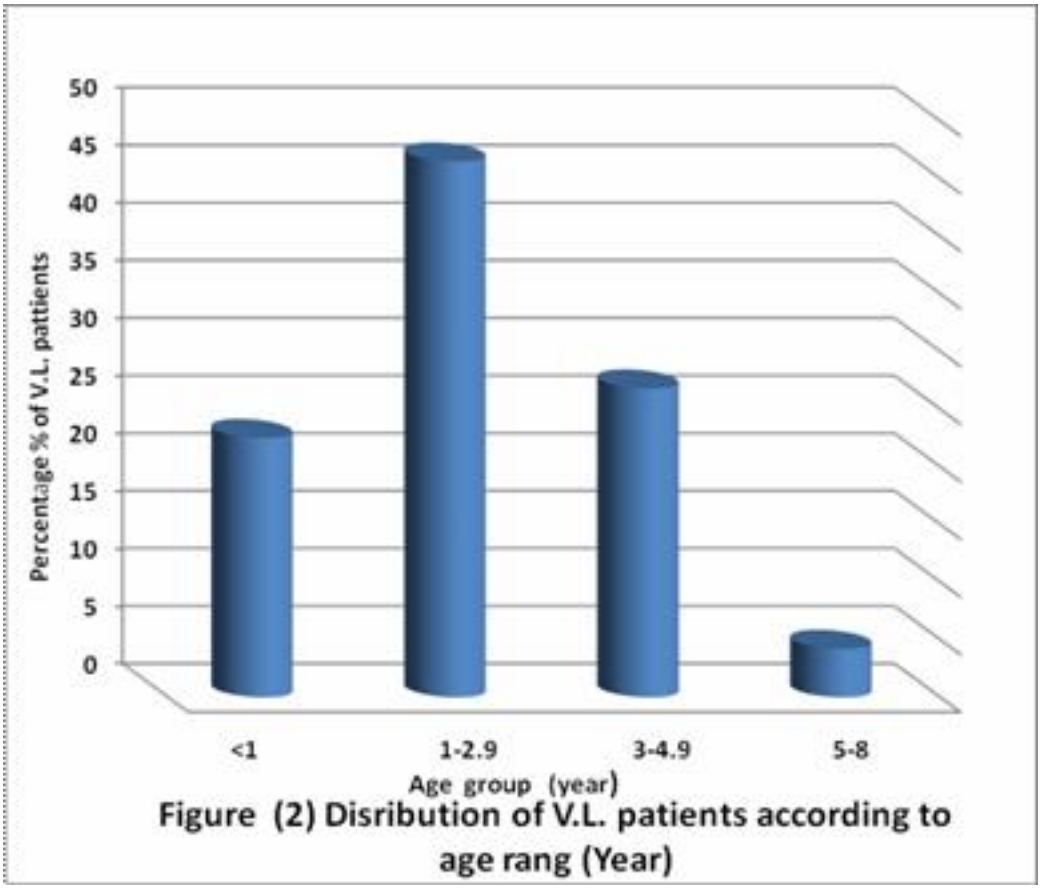

Figure (2) Disribution of V.L. patients according to age rang (Year)

تقييم اختبار الاليزا النقطي Dot. ELISA في تشخيص الأطفال المصابين باللاشمانيا الاحشائية

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الخلاصة

أجريت هذه الدراسة لأول مرة في العراق لتقييم فائدة اختيار الاليزا النقطي Dot.ELISA للكشف عن أضداد *Leishmania donovani.* ل 73 طفلا"بعمر اقل من 8 سنوات المتوقعة إصابتهم بمرض اللاشمانيا الاحشائية.V.L و 60 مصلا لأشخاص أصحاء، و 57 مصلا لمصابين بأمراض أخرى غير V.L .
(Amoebiasis, Giardiasis, Toxoplasmosis ,Schistosomiasis , Hydatidosis, Ascaiasis , Lupus Erythromatosus , Viral Hepatitis , and Cutaneous Leishmaniasis).

أوضحت النتائج إن الإصابة في مجموعة الذكور كانت اعلى من الإصابة في مجموعة الإناث ، وإن نسبة انتشار المرض كانت الأعلى ضمن الفئات العمرية 1– 2.9 3– 4.9 سنة وبفارق ذي دلالة معنوية عالية بالمقارنة مع الفئات العمرية الأخرى (P>0.01). وجدت أضداد *Leishmania donovani* في 71 عينة (من 73) متوقعة إصابتهم بالمرض . التفاعل تصالبي وجد مع أمصال مرضى داء الذئبة الاحمراري الجهازي ولم نجد هذا مع الأمراض الأخرى . بلغت حساسية الاختبار97.3%، والخصوصية 96.3 %.القيم التنبوئية الايجابية والسلبية للاختبار 96 % و 97.4 % على التوالي.

اختبار Dot. ELISA بسيط ، سريع ، رخيص الثمن، وذو حساسية وخصوصية ونظرا لذلك نوصي باستعماله في التشخيص المصلي بداء اللشمانيا الاحشائية.