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Original Article

Sero-epidemiological study of Visceral Leishmaniasis in Basrah, Southern Iraq

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Abstract

Objectives: To study selected epidemiological aspects of visceral leishmaniasis, assess direct agglutination test (DAT) as a diagnostic method and the sero-epidemiological prevalence of the disease among apparently healthy children in Basrah, Iraq.

Methods: This prospective study included 146 children suspected of visceral leishmaniasis who were admitted to Basrah Maternity and Children Hospital and Basrah General Hospital from November 2004 till November 2005 and 37 serum samples that were collected from patients with different diseases considered in the differential diagnosis of visceral leishmaniasis.

In addition, 1000 apparently healthy children were randomly selected for the sero-epidemiological survey. Direct agglutination test was done for all of them.

Results: Out of 146 suspected visceral leishmaniasis cases, 124 (84.9%) were proved by the examination of bone marrow aspirate, 132 (91.1%) were positive by direct agglutination test (DAT) and only 3 (2%) were positive by imrnunochromatographic strip test. The sensitivity and specificity of DAT were (100%), with a cut-off point of 1:800.

In the in-patient group children less than 2 years of age were mainly affected. The highest frequency of disease was reported in July, 24 cases (18.8%).

Sand flies were recorded in the environment of all sero-positive cases (100%) in each group, stray dogs and wild canines were present in (75.7% and 15.5%) in in-patients group compared to (69% and 22.5%) in sero-epidemiological group, respectively. Low maternal education was present in a significantly higher frequency among sero-positive cases.

Conclusions: Direct agglutination test can be used as a screening tool for visceral leishmaniasis on a wide range in endemic areas, with a high sensitivity and specificity (JPMA 60:464; 2010).

Introduction

Visceral leishmaniasis (VL) continued to be an important public health problem in countries of the Eastern Mediterranean Region. A fourfold to six fold rise in the number of cases in 1991 compared to 1990 was observed in Iraq, namely 576 cases of visceral leishmaniasis in 1990 compared to 3713 cases of visceral leishmaniasis in 1991. However, other factors such as population movement and the destruction of health and vector-control facilities during the war contributed to the outbreak of leishmaniasis in Iraq.[1]

In Basrah, only 41 cases were recorded during the period 1971- 1984[2] and l47 cases for the period of 1993-1997.[3] According to the data of CDC Surveillance Unit/ Primary Health Care Department/ Basrah, the number of cases that were reported in 2004 and 2005 was 608; about 210 (34.5%) of them were from Al-Qurna district.[4]

The clinical diagnosis of visceral leishmaniasis is complex because it's clinical features are shared with other commonly occurring diseases, such as malaria, typhoid fever and tuberculosis; many of these diseases can be present along with visceral leishmaniasis as co-infection.[5]

The visualization of the amastigote form of the parasite by microscopic examination of aspirates from lymph nodes, bone marrow or spleen is the classical confirmatory test for VL. Although the specificity is high, the sensitivity of microscopy varies, being higher for spleen (93-99%) than for bone marrow (53-86%) or lymph node (53-65%) aspirates.[6]

In 1988, a modified Direct Agglutination Test (DAT) was reported to be useful in the diagnosis of visceral leishmaniasis and is being used in several endemic countries.[7] DAT in various studies has been shown to be (91-100%) sensitive and (72-100%) specific.[8-10]

Because of the conditions prevail1ing in areas of endemicity, sophisticated method cannot be employed on a wider scale. There is a need for a simple, rapid and accurate test with good sensitivity and specificity which can be used without any specific expertise.[5] A promising ready-to-use immunochromatographic strip test is based on, a recombinant antigen rK39, which has been developed as a rapid test for use in difficult field conditions.[5]

This study was carried out to evaluate selected epidemiological aspects of visceral leishmaniasis, assess direct agglutination test (DAT) as a serological diagnostic method and study the sero-epidemiological prevalence of the disease among apparently healthy children in 3 areas in Basrah with different endemicity using the DAT method.

Patients and Methods

This study is a prospective study that was carried out over 16 months (from the first of November 2004 till the end of February 2006). The study included two major groups of children (patients and apparently healthy children)

In-Patients Group: This group was subdivided into 2 subgroups (Subgroup one & two):

Sub-Group One: This included children with clinical features suggestive of visceral leishmaniasis who were admitted to paediatric wards of Basrah Maternity and Children Hospital and Basrah General Hospital from the first of November 2004 till the first of November 2005.

Sub-Group Two: This sub-group included patients with diseases other than visceral leishmaniasis. The cut-off point, sensitivity and specificity of the visceral leishmaniasis DAT test, was used in this study.

It included; thirty seven patients with different diseases according to the differential diagnosis of visceral leishmaniasis.[11] Ten patients with leukemia, 7 patients with tuberculosis, 5 patients with typhoid fever, 6 patients with cutaneous leishmaniasis, 4 patients with Brucellosis, and 5 patients with Hepatitis A.

Apparently Healthy Children (Sero-Epidemiological Group):

This included ten apparently healthy children (randomly selected) that had visited 3 primary health care centers in Basrah governorate for vaccination, from the period of November 2005 to February 2006, their ages ranged from 2 months to 9 years.

The geographical areas of health centers were selected according to the endemicity of visceral leishmaniasis in Basrah according to the data that were obtained from primary health care department of Basrah governorate for the period from 2004-2005.[4]

Incidence rate = No. of infected cases in area / Total No. of population in this area X 100,000.

Accordingly, the low, moderate ,and high incidence rate areas were selected depending on visceral leishmaniasis cases registered in 2005; First Basrah Sector (5%), Al-Hartha (10.8%) and Al-Qurna (36.6%) respectively.

Five hundred children were randomly selected from children attending Al-Razi Health -First Basrah sector (low endemic area), while two hundred- fifty children each were randomly selected from children attending both Al-Hartha Health Center- Al-Hartha Sector (moderate endemic area) and Al-Dair Health Center -Al-Qurna Sector (high endemic area).

Data collection included full information of patients and apparently healthy children, obtained from one of the

parents, including mother's and father's education. Full address, building material, presence or absence of wild canine, presence or absence of stray dogs, and domestic animals. Also, presence or absence of water collections, and whether the mosquito net was used or not. In addition, clinical data were recorded and physical examination was done of each patient. All patients with confirmed or suspected visceral leishmaniasis were sent for complete blood count and blood film. In addition, results of bone marrow examination were recorded. The importance of the test was explained to parents and an informed consent was obtained before recruitment in the study.

Sampling and Examination was done by collection of two ml. of blood which was allowed to clot, at room temperature then centrifuged at 3000 r.p.m. for 5 minutes and part of separated serum samples were used for Immunochromatographic dipstick test, the remaining serum was stored at -20°C until used in (DAT), (this step; i.e serum for DAT, included the two subgroups of patients. Children with suspected or confirmed visceral leishmaniasis were 146 and 37 patients with other different diseases).

One thousand blood spot samples were collected on Whatman filter papers from apparently healthy children, left to dry and stored in cooled dry place at -20°C until used for DAT.[7] Bone Marrow examination was done for all in-patients suspected to have visceral leishmaniasis.

Measurement of Cut-Off Point:

Thirty seven serum samples that were collected from patients with different diseases that are considered in the differential diagnosis of visceral leishmaniasis, 10 serum samples from patients confirmed to have visceral leishmaniasis and another 10 serum samples from apparently healthy control group .

Titers from 1:100 to 1:3200 were used to define the cut-off point; the point (or dilution) at which the serum of all diseases other than visceral leishmaniasis gave negative results and did not show reaction.

Determining the Sensitivity and Specificity of DAT:[12,13]

The sensitivity of DAT was assessed with sera from confirmed visceral leishmaniasis patients (No.10); positive bone marrow examination. Sera of apparently healthy controls (no.10) and sera of patients with other diseases (no.37) were used to determine the specificity of DAT.

Immuno-chromatography Dipstick Assay (Kala-azar DetectTM Rapid Test) was used to detect antibodies to leishmania donovani complex. A positive result indicates that the Kala-azar DetectTM dipstick detected antibodies to members of leishmania donovani complex.

The Direct Agglutination Test (by Koninklijk Institute Voor De Tropen- Royal Tropical Institute/ Amsterdam) was carried out using leishmania freeze-dried antigen with a parasite density of (5x 107 parasite per ml).

The chi-square (X2) test was used as a test of significance. Differences were recorded as significant whenever probability (p) was less than 0.05.

SPSS - version 10.0 windows computer programme was applied for multivariate regression analysis.

Results

Results of Different Diagnostic Methods: This study included subgroup 1, (146) in-patient children suspected of having visceral leishmaniasis. Eighty six were males and 60 were females, their ages ranged from 2 months to 12 years; mean age was 5.82 ± 4.57 years. Those positive for Leishmann donovani bodies on bone marrow examination were 124 (84.9%), while 132 (91.1%) were positive by DAT and 3 (2%) were positive by dipstick test.

Results of Direct Agglutination Test (DAT): The results of the DAT reactions for different diseases involved in the differential diagnosis and in different dilutions are presented in Table-1. Sera from various diseases (no=37) and sera from apparently healthy control (no=10), gave positive results at titers 1:400, and show cross reactivity with visceral leishmaniasis antigen (DAT) in these titers. All the 10 serum samples from parasitologically proven cases of visceral leishmaniasis gave positive results at titers ≥ 1:800, and there were no cross reactions with other diseases and healthy control in these titers. This table demonstrates that the sero-positivity at 1:800 is considered as the cut-off point for the DAT in the diagnosis of visceral leishmaniasis, because in this dilution, serum of all diseases other than visceral leishmaniasis became negative and did not give any reaction. According to the cut-off point (1:800), the sensitivity and specificity of the DAT were (100%).

Geographical distribution: All the 132 sero-positive cases were from southern governorates of Iraq, 89(67.4%) cases were from Basrah, 36 (27.3%) cases were from Maysan, 6 (4.5%) cases were from Thi-Qar, and one case (0.7%) was from Baghdad. Among the 89 sero-positive cases of visceral leishmaniasis from Basrah, Al-Qurna sector had the highest rate of positivity 23(25.8%),followed by the first Basrah sector and Al- Hartha sector (15.7%), second Basrah sector (12.4%), Abu-Al-Kassib (10.1%), Al-Zubair (9%), Al-Madina (7.9%) and the lowest one was in Shatt-Al-Arab sector 3(3.4%).

Among all sero-positive visceral leishmaniasis cases included in this study, the highest frequency of visceral leshmaniasis was reported in July, 24 cases (18.8%), while

Table-1: Cut-off point value for DAT test.

Disease	No.	<1:100	1:100	1:200	1:400	1:800	>1:800
			Inpatient group				
Sub-group 1:							
Visceral leishmaniasis	10	-	-	-	-	4	6
Sub-group-2:							
Cutaneous leishmaniasis	6	-	3	2	1	-	-
Healthy Control	10	5	2	2	1	-	-
Leukaemia	10	-	-	-	-	-	-
Brucellosis	4	1	2	1	-	-	-
Typhoid Fever	5	1	2	1	1	-	-
Tuberculosis	7	2	3	1	1	-	-
Hepatitis	5	2	2	1	-	-	-
			Sero-epidemiological group				
Apparently healthy control	10	5	2	2	1	-	-

the lowest number of cases was reported in November, 3cases (2.2%).

The outcome of the sero-positive patients that were admitted to the hospitals was recorded. About half of these cases 65 (49.2%) improved and discharged well from the hospitals, 42 (31.8%) were discharged on the family responsibility with unknown outcome, while 15 (11.4%) patients died during the period of hospitalization. Ten patients (7.6%) were admitted again after being discharged from the hospital.

Multivariate regression analysis has revealed that three variables ; younger child ($p< 0.05$) , male sex ($p<0.001$), and high DAT titer ($p<0.001$) were found to be significant predictors of death and relapse .

Sero-epidemiological Group: One thousand apparently healthy children (509 were males and 491 were females) were randomly selected from 3 health centers, of different disease endemicity, in Basrah , 2 months - 9 years old. These were Al-Razi, Al-Hartha and Al-Dair primary health centers.

A total of 200 out of 1000 individuals (20%) demonstrated DAT positive results. The cut-off value for DAT employed in this study was 1:800. Children from Al-Dair gave the highest sero-positivity rate, 87 out of 250 (34.8%), while those from Al-Razi gave the lowest, 45 out of 500 (9%). In the third health care center, (Al-Hartha), 68 children (27.2%) gave positive results.

Comparison between In-Patients Group and Sero- Epidemiological Group:

Age and Sex Distribution:

Table-2 demonstrates that the highest rate of sero-positivity was in the first two years of life. In the patients group, infants and children below 2 years of age were mainly affected (74.3%). The percentage was decreased sharply as the age increased. Among sero-epidemiological studies, although the highest seropositivity was also in age groups below 2 years (45.4%), there were no significant differences between different age groups. There was statistically significant differences between in-patient and sero-epidemiological groups ($p<0.05$) in relation to age groups. In addition, there was no significant difference ($p>0.05$) in the frequency of the disease between males and females in all age groups.

Relation to Different Environmental Variables of the Sero-Positive Cases:

Sand flies were recorded in the environment of all sero-positive cases (100%) in each group, stray dogs and wild canines were present in (75.7% and 15.5%) in in-patients group compared to (69% and 22.5%) in sero-epidemiological group, respectively, Table-3. Rodents were also recorded in the environment of each group in high percentages (79.5% and 69%). Statistically there was significant difference

Table-2: Comparison between age and sex distribution of sero-positive cases of inpatient and sero-epidemiological groups.

Age* (years)	In-patient group (No. 132)		Sero-epidemiological group (No. 200)	
	No.	(%)	No.	(%)
< 1 year	54	(41)	66	(21.7)
1-	44	(33.3)	43	(23.7)
2-	13	(9.8)	27	(20)
3-	5	(3.8)	17	(15.4)
4-	2	(1.5)	12	(14.6)
5-	4	(3.0)	11	(20)
6-	5	(3.8)	11	(18.3)
7-	1	(0.8)	7	(16.6)
8-	2	(1.5)	6	(19.3)
9-12	2	(1.5)	0	(0)
Sex				
Male	78	(59)	101	(50.5)
Female	54	(41)	99	(49.5)

*$p < 0.05$ between in-patient and sero-epidemiological group, in relation to age.

($p<0.05$) concerning the presence of domestic animals, rodents, tree and crops and in water collections. But there was no significant difference regarding the presence of canine animals, stray dogs and sand flies, Table-3.

Table-3: Distribution of different environmental variables and parental educational level of sero-positive cases among in-patient and sero-epidemiological groups.

Variables	In-patient group (No. 132)		Sero-epidemiological group (No. 200)	
	No.	(%)	No.	(%)
Domestic animals*	58	(43.9)	43	(21.5)
Stray dogs	100	(75.7)	138	(69)
Wild canine	20	(15.5)	45	(22.5)
Rodents*	105	(79.5)	138	(69)
Sand flies	132	(100)	200	(100)
Trees and Crops*	69	(52.2)	60	(30)
Water collections*	97	(73.4)	90	(45)
Educational level				
Father Education				
Illiterate & just literate	67	(50.8)	105	(52.5)
Primary & Intermediate education	43	(32.6)	62	(31)
Secondary & Higher education	22	(16.6)	33	(16.5)
Mother Education*				
Illiterate & just literate	100	(75.8)	110	(55)
Primary & Intermediate education	26	(19.7)	64	(32)
Secondary & Higher education	6	(4.5)	26	(13)

* P value < 0.05.

Comparison According to Parental Education:

Higher percentage of sero-positive children in both groups have fathers who were illiterate or just literate (50.8% and 52.5%), Table-3. Statistically the difference was not significant , $p>0.05$.

Concerning maternal education, most children in the in-patient have mothers with low educational level (75.7%), compared to (55%) in the survey, Table-3. There was significant difference between two groups according to maternal education level ($p<0.05$).

Discussion

Leishmaniasis is recognized as an important public health problem in some countries of the Eastern Mediterranean Region owing to its considerable impact on morbidity, which impose a heavy burden on national health.[1]

In the current study (84.9%) were bone marrow positive, while, this finding is in agreement with Al-Shanawi study where (83.4%) of clinically suspected cases of visceral leishmaniasis were positive by bone marrow examination.[14] In other studies, bone marrow stained smear examination was the least sensitive method for detection of visceral leishmaniasis (67.1-70%).[15-17]

Much progress has been made in the development of less invasive tests to assist in the diagnosis of visceral leishmaniasis. In this study, DAT and Immunochromotography dip stick of rK39 (Kala-azar dip stick) were used. DAT gave a sero-positivity in (91.1%) with a sensitivity and specificity of 100%. In other studies the sensitivity of DAT was ranging between (80-100%),[12,18] while the specificity was ranging between (96.4-100%).[12,18,19]

A titer of 1:800 was considered as a cut-off point for DAT. In other studies 1:1600 and 1:3200 were considered as cut-off point because Trypanosomiasis are closely related to Leishmania organism; so serum samples from patients with Chaga's disease or African Trypanosomiasis cross reacted in the DAT in a dilution of 1:800.[7,12] As these diseases are not recorded in Iraq, there is no problem in reducing the cut-off point of DAT to 1:800.

Immunochromotography dip sticks with rK39 leishmanial antigen was positive only in 3 (2.0%), in comparison with other studies, the sensitivities of rK39 antigen strip test ranged from (67-100%). The highest sensitivity (100%) was reported in patients from India and Nepal,[20,21] while this test has low sensitivity (67%) in patients from Sudan.[22]

There are several hypotheses that could explain this regional variation of the results of the rK39 antigen strip test. First, there may be differences in the test accuracy between subspecies of the Leishmania donovani complex. Similarly, within these subspecies, there may be regional differences as a result of variations in the K39 antigen. Another possible explanation involves genetic differences in individual patients or in racial subgroups. In Iraq, the main causative agent of visceral leishmaniasis is Leishmania infantum,[1] so the low sensitivity of this test in Iraq may be attributed to the low level or absence of this antigen (K39) in Leishmania infantum species.

Regarding cases from Basrah, (25.8%) of these cases were from the northern parts of Basrah (Al-Qurna district). Al-Qurna is a rural area with high humidity of the soil and there is dense vegetation, these factors probably provide a suitable environment for breeding of sand flies. Moreover, high density of stray dogs and wild canines are present in the surrounding areas which may act as reservoir hosts.

Rural areas (Al-Dair health care center) had the highest sero-positivity rate (34.8%) compared with urban areas (Al-Razi) (9%). These results reflect a lower standard of living in the rural area than in urban area, and this leads to an

increase risk of transmission of the disease in the rural areas. This can be attributed to the life style and the house building material had an effect of increasing transmission of the disease. Poorly constructed house is easily invaded by the sand flies and it is a preferred place for breeding, so this variable is an important risk factor, since a study showed that transmission usually occurs in and around houses.[23]

Most of patients admitted to hospitals were less than 2 years age and the sero-positivity was decreased as age increased. While in the survey study all age groups had a risk to be infected with visceral leishmaniasis, this is the first time such a result is recorded in Iraq. Other studies concluded that the sero-positivity rate decreased with increasing of age and the most risky group was that of less than 3 years old in both hospitalized patients and sero-epidemiological studies.[5,15]

Exposure of children to sandflies, domestic animals, stray and wild canines and other factors in the envirnoment may help to build up acquired immune response against the disease, leading to high titer of DAT in these children. Pampiglione etal, suggested that it was probable that clinically evident cases, form the tip of the iceberg in the prevalence of infection.[24]

As low maternal education was present in a significantly higher frequency in the in-patient group compared to those included in the survey, parental education is important to improve the socioeconomic status and standard of living, and this may confirm the importance of health education in the control programme of visceral leishmaniasis.

The use of DAT in present study will open the door for other researchers to use this test as a screening tool for visceral leishmaniasis on a wide range in endemic areas. DAT is (cheap in price, simple test, not invasive, with a high sensitivity and specificity, easy-to-perform and does not require specialized equipment).

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