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Indirect Fluorescent antibody test for serodiagnosis of visceral leishmaniasis: An epidemiological study in Iraq.

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ABSTRACT

Fourteen thousand, five hundred and two sera of patients from sixteen governorates in Iraq ,suspected to be parasitized by Leishmania donovani, have been tested during the period January 2000 to December 2002, by indirect fluorescent antibody test (IFAT) using a leptomonad antigen. A total of 5558(38.5%) cases of them were found positive for IFAT for visceral leishmaniasis (VL). The most common age group infected with VL was between 1- 5 years; they represented 5390 (96.97 %). The highest ratio of VL infections was in February and January; they represented 510 positive cases in February 2000, 417 in January 2001 and 513 in February 2002. It was seems that Wasit governorate had the highest percentage of infections; it was 35 %, 29.6 % and 33 % in 2000, 2001 and 2002 respectively, then it follows by Diala, Babil and Baghdad governorates.

Introduction

The causative agent of visceral leishmaniasis (VL) or kala- azar in majority of endemic countries in the eastern Mediterranean region is Leishmania donovani.VL is widely distributed and it is suspected to occur in 47 countries. Within a geographic area, there is often patchy distribution delimited by proper vector habitat and suitable reservoir hosts (1).

Epidemic of classic VL leading to thousand of deaths are ongoing in Brazil, India and Sudan. A marked increase in cases is often associated with an influx of nonimmune populations into newly cleared agricultural areas or population expansion into previously unsettled areas surrounding cities (2).

Several species of sand fly are incriminated as vectors of VL. There are still important foci where little is known of possible vectors, and new species of vectors are being discovered (3).VL is basically a disease of healthy infants and adults. However, in the last decade an increasing number of cases of kala-azar in immunocompromised patients have been reported with emphasis on a typical manifestation of the disease (4, 5).

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The results of studies reported by Korzeniewski and Olszanski (6) concerning the incidence of leishmaniasis among soldiers of stabilization forces serving in Iraq in the years from 2003-2004.

Diagnosis of leishmaniasis is based on clinical, serological and parasitological identification. Some countries use improved serodiagnostic procedures for diagnosis of VL like the enzyme- linked immunosorbent assay (ELISA), direct agglutination test (DAT), indirect fluorescent antibody test (IFAT) , or molecular techniques like polymerase chain reaction (PCR) technique (7, 8).

Difficulties in clinical and parasitological diagnosis of VL still exist. The signs and symptoms of the disease in areas with multiple morbidity may not be sufficiently specific to differentiate VL from other disease. Therefore, the majority of countries presently apply serology techniques for diagnosis of VL. The present study describes the results of an indirect fluorescent antibody test for the detection of VL in more than 14000 patients from different parts of Iraq.

Methods

Study group

A total of 14502 patient's sera were included in this study from sixteen Iraqi governorates as shown in table (4). They have been collected in the central health laboratories in Baghdad through January 2000 to December 2002. Sera from normal individuals known not to have undergone actual parasitism or previous exposition to L. donovani infection were used as controls

Serology test

The indirect fluorescent antibody test (IFAT) was performed following the same procedure described in previous study by Mayrink, et al., (9). The materials used in the test were provided by (Biomerieux).

First: Antigens preparation

The antigens were prepared from L. donovani leptomonads grown in semisolid medium at 22○C. They were washed three times in phosphate buffer saline (PBS) pH 7.2 by centrifugation at 1500 rpm for 10 minutes.

A small drop of final sediment was put on each circle of the slide (On each slide 10 circles). The smears were then dried at room temperature, and then stored at 4○C without the use of any fixative.

Second: Technical procedures

In general, the following steps that have been proposed by Mayrink, et al., (9) were performed.

10µl of diluted serum for each patient were added into one circle of the slide which contains the antigen.

The preparation slides were kept at 37○C for 30 minutes.

The smears were submitted to 10 minutes bath in PBS (pH7.2) then 5 minutes in distilled water and subsequently dried up.

Diluted anti- human globulin labeled with fluorescein isothiocyanate (Ig – FITC) is then applied.

Incubation, washing and drying as in step 2 and 3.

Sera were titrated to determine the highest dilution giving optimum fluorescence with homologous antigens.

Titers ranged from 1: 16 to 1: 1024. Through out all tests, sera were used at dilutions corresponding to their respective fluorescent titers; most of them show the highest fluorescence at the dilution 1: 64.

Third: Microscopic examination

Fluorescent microscopy was accomplished by using a (CETI- Germany) microscope assembled with an (USH- 102D) ultra violet lamp.

Statistical analysis

Data in terms of observed numbers and analyzed by using chi- square ($\chi 2$) test. P value < 0.05 was considered statistically significant.

Results

During January 2000 to December 2002, the Central Health Laboratories in Baghdad identified 14502 cases by IFAT test suspected to be infected with kala azar from different governorates; most of them had fever, enlargement of the liver and spleen.

The fluorescent antibody was found to fix mainly on the leptomonad membrane and flagellum. The negative reaction did not demonstrate any fluorescence although the leptomonads could identified as a very pale yellowish shadow.

Out of 14502 cases, 5558 (38.5 %) cases were positive for IFAT test for VL distributed between the three years, as shown in (table 1). The most common age range infected with VL was 1-5 years; they represent 96.8 %, 97.8 % and 96.3 % for the years 2000, 2001 and 2002 respectively. The infections were much lower in the other ages (table 2). There were significant differences between them ($P < 0.05$).

(Table. 3) show the monthly distribution of VL infections through the three years. They were 510 positive cases in February 2000, 417 in January 2001 and 513 in February 2002.

In general most infections concentrated in three months: January, February and March. These results showed highly significant differences between infections in different months ($P < 0.05$).While (table. 4) show the distribution of VL infections in different governorates in Iraq. It was seems to be that Wasit governorate had the highest percentage of infection; it was 32.4 % in 2000, 30.9 % in 2001 and 30.1 % in 2002, then Diala, recorded 22.64% of infections in 2000, 19.6% in 2001 and 20.6%

in 2002, and Babil recorded 13.7% of infections in 2000, 16.4% in 2001 and 15.3% in 2002 while Baghdad recorded 12.5% of infections in 2000, 11% in 2001 and 11.2% in 2002. . The other governorate shows low incidence of VL infections. There were significant differences between them ($P < 0.05$).

Discussion

Mayrink, et al., (9) showed that Fluorescent antibody titers were higher than those observed with the complement fixation test, because IFAT is generally performed by using a homologous antigen, so the antibodies detected are specific, while complement fixation test performed by using a heterologous antigen. Thus the antibodies detected are quite non- specific. Duxbury and Sadun, (10) suggested that IFAT might be very useful for diagnosing cutaneous leishmaniasis CL and VL from the Mediterranean type, they also reported that kala azar serum gave strongly positive reactions when tested against antigens from leishmanial bodies and leptomonads of L. donovani.

In the present study, the incidence of VL in patients aged between 1-5 years was higher as compared to other ages. The decrease in incidence with age may be due to the development of immunity by previous infections. These findings are within agreement with those reported by (11).

Infections of VL tended to increase in November and December and reach a maximum in January and February. The incidence rate of infection then starts to decline from May and reaches its lowest point in August and September. This variation in seasonal peak could be due to the existence of various dominant reservoir species in these areas as well as to the activity of the sand flies. The differences in monthly distribution of VL patients might also be related to the development of female insects and their requirement of blood during their life cycle for the maturation and development of eggs, especially in spring season. The lapse of time between when the patient was bitten and the appearance of the symptoms might be related to the long incubation period of leishmaniasis (2-4 months) (1).

There are many factors that play an important role in the presence and distribution of VL in Wasit, Diala and Babil, including the presence of animal reservoirs such as rodents, dogs, and the use of clay to build some of the houses in villages in these areas. Furthermore, these governorates contain agricultural areas that district attracts and harbors many kinds of insects, and there population work long hours in the farms where they are more exposed to insects bites. Also, VL in our country may primarily affect families of farmers and nomads who they are chiefly exposed to night biting sand flies. The presence of high gerbil population densities in these areas may be blamed as reservoir of infection that is supported by the crops for which the irrigation canals had been constructed. Furthermore, the canal embankments serve as densely populated and favored rodent and sand fly infestation areas (11).

In addition, a shortage of specific drugs for the treatment of leishmaniasis can potentially contribute to the intensity of transmission in the foci of anthroponotic forms of the disease, and subsequently to an increase in the morbidity and even mortality among populations at risk of infection. Generally all humans serve as reservoir hosts during epidemics of anthroponotic forms of infection, and if untreated, the severity of the disease will increase and prove fatal in case of a kala azar epidemic. The lack of diagnosis facilities and drugs for treatment have been among the possible contributing factors to the epidemic of kala azar in Sudan between 1988 and 1993, and the outbreak of kala azar and anthroponotic CL in Iraq after the gulf war and subsequent economic embargo in 1991(2).

The emergence of leishmaniasis in some foci may be the result of interruption of previously applied methods of control, like insecticide spraying on early diagnosis and treatment of positive cases. It is believed that reduction in insecticide spraying for malaria control contributes to the increase in the population of synanthropic sand flies and results incidence of the disease in some endemic foci of VL and CL (2, 7).

It is worth mentioning that such infections are still going on since most parasitic infections between American military forces who had been deployed to Afghanistan and Iraq were malaria and leishmaniasis. Among American troops serving in Iraq, between 2003 to march 2004, 653 cases of CL and 2 cases of VL.

According to the U.S. sources, the number of infected American soldiers could have been higher, from 750 to 1250 or even more, what made up to nearly 1% of U.S. troops serving in Iraq in 2003- 2004 (6, 12).

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Table 1: The yearly distribution of visceral leishmaniasis in Iraq.

Infection Years	Tested cases	Positive cases	Percentage
2000	4600	1934	42%
2001	4345	1710	39.3%
2002	5557	1914	34.4%
Total	14502	5558	38.5%

Table 2: The distribution of visceral leishmaniasis infection between age groups.

Year Age	2000	2001	2002	Total
1 – 5	1873 (96.8 %)	1673 (97.8%)	1844 (96.3%)	5390 (96.47)
6 – 10	47 (2.4%)	30 (1.75%)	46 (2.4%)	123 (2.213)
Above 11	14 (0.7%)	7 (0.4%)	24 (1.2%)	45 (0.80)
Total	1934	1710	1914	5558
Statistical analysis χ^2Cal = 10.795 , χ^2Tab = 9.49				

Table 3: The monthly distribution of visceral leishmaniasis infection from 2000 -2002 years in Iraq.

Year Month	2000	2001	2002	Total
January	441 (65%)	417 (62.4%)	414 (56.6%)	1272
February	510 (61.5%)	317 (52.3%)	513 (81.8%)	1340
March	240 (40.4%)	284 (56.1%)	329 (41.1%)	853
April	140 (28.5%)	171 (46.7%)	152 (25%)	463
May	90 (25.7%)	131 (34.9%)	175 (38.1%)	396
June	85 (28.4%)	27 (8.4%)	93 (25.1%)	205
July	45 (18%)	13 (5.2%)	42 (13.3%)	100
August	34 (18.2%)	12 (5.6%)	18 (7.8%)	64
September	46 (26.2%)	22 (11%)	23 (10.1%)	91

October	**58 (26.8%)**	**43 (18.4%)**	**44 (15.3%)**	**145**
November	**73 (31%)**	**52 (27.6%)**	**83 (21.8%)**	**208**
December	**172 (57.7%)**	**221 (52.1%)**	**28 (5.3%)**	**421**
Total	**1934**	**1710**	**1914**	**5558**
Statistical analysis	**χ^2 Cal = 323.395 , χ^2Tab = 33.92**			

Table 4: The distribution of visceral leishmaniasis infections in different governorates in Iraq.

Years	**2000**	**2001**	**2002**	**Total**
Wasit	**628 (32.4%)**	**530 (30.9%)**	**577 (30.1%)**	**1735**
Diyala	**438 (22.64%)**	**336 (19.6%)**	**395 (20.6%)**	**1169**
Babil	**265 (13.7%)**	**282 (16.4%)**	**294 (15.3%)**	**841**
Baghdad	**242 (12.5%)**	**188 (11%)**	**216 (11.2%)**	**646**
Al-Qadisiyyaah	**145 (7.4%)**	**156 (9.1%)**	**182 (9.5%)**	**483**
Al-Anbar	**68 (3.5%)**	**57 (3.3%)**	**73 (3.8%)**	**198**
Salah Al-Din	**56**	**47**	**64**	**167**
	(2.8%)	**(2.7%)**	**(3.3%)**	
Thi Qar	**28 (1.4%)**	**43 (2.5%)**	**38 (1.9%)**	**109**
Maysan	**18 (0.9%)**	**19 (1.1%)**	**22 (1.1%)**	**59**
Karbala	**11 (0.5%)**	**7 (0.4%)**	**10 (0.5%)**	**28**
Al-Muthanna	**13 (0.6%)**	**15 (0.8%)**	**13 (0.6%)**	**41**
Al-Basrah	**1 (0.05%)**	**2 (0.11%)**	**2 (0.1%)**	**5**
Kirkuk	**4 (0.2%)**	**8 (0.46%)**	**3 (0.15%)**	**15**
Al-Najaf	**13 (0.6%)**	**20 (1.16%)**	**25 (1.3%)**	**58**
Al-Sulaymaniyyah	**1 (0.05%)**	**-**	**-**	**1**
Ninawa	**3 (0.15%)**	**-**	**-**	**3**
Total	**1934**	**1710**	**1914**	**5558**
Statistical analysis	**χ^2 Cal = 45. 24 , χ^2Tab= 43.77**			

استخدام فحص الاجسام المضاده الومضائيه غير المباشر في التشخيص المصلي لداء اللشمانيا الاحشائيه: دراسه وبائيه في العراق

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الخلاصه:

أجري فحص 14502 مصل لمرضى من ستة عشر محافظه في العراق مشكوك باصابتهم بطفيلي اللشمانيا الاحشائيه خلال الفترة من كانون الثاني 2000 الى كانون الاول 2002 , وذلك من خلال فحص الاجسام المضاده الومضائيه غير المباشر باستخدام مستضدات الطور السوطي.ان ما مجموعه 5558 (38.5 %) حاله منهم اظهروا نتيجه موجبه لفحص الاجسام المضاده الومضائيه غير المباشر للشمانيا الاحشائيه .لقد وجد بأن الاعمار الاكثر عرضه للاصابه باللشمانيا الاحشائيه تقع بين 1 – 5 سنوات وكانت تمثل 5390 (96.97 %) حالة من المجموع الكلي. لقد ظهرت اعلى نسبه من الاصابه في شهر شباط و كانون الثاني وكانت تمثل 510 اصابه في شهر شباط لسنة 2000 و 417 اصابه في شهر كانون الثاني لسنة 2001 و 513 اصابه في شهر شباط لسنة 2002 .كما تبين النتائج النسبه الاعلى للاصابه في محافظة واسط اذ بلغت 35 %, 29.6 % و 33 % في سنة 2000, 2002 و 2002 على التوالي, ويليها كل من محافظة ديالى, بابل و بغداد.