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A Cloned Antigen (Recombinant K39) of *Leishmania donovani* Diagnostic for Visceral Leishmaniasis in Human

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أستخدام التقنية الوراثية للشريط rK39في تشخيص مرض اللشمانيا ألأحشائية

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الخلاصة :-

يعتبر مرض اللشمانيا الحشوية أو الكالازار من ألأمراض الطفيلية التي يسببها طفيلي أبتدائي يدعى لشمانيا دونافاني . يمكن تشخيص المرض مختبريا من خلال التحري عن ألأجسام المضادة في الدم اوالمصل عن طريق فحص جمعت rK39 الذي أثبت نجاحه عن طريق حساسيته ونوعيته العاليتين. ١٠٠عينة دم بشرية من أشخاص مصابين بمرض الكالازاروقسّمت الى أربع مجاميع عمرية أثبتت نتائج الدراسة أن أعلى ألأصابات كانت ضمن الفئة العمرية دون السنة (٦٠%) وأدناها ضمن الفئة العمرية فوق ٤٠ سنة(١%) كما بيّنت الدراسة ان الذكور(٦٢%) كانوا أكثر أصابة من ألأناث(٣٨%) وأن نسبة الذكور / ألأناث هي ١/١.٦ .

ABSTRACT

Visceral leishmaniasis (VL) or kala-azar is caused by an intracellular protozoan parasite of the *Leishmania donovani* complex and is considered as one of the most neglected diseases.To detect IgG antibody in the serodiagnosis of visceral leishmaniasis (VL), a recombinant antigen rK39, which is part of a *Leishmania donovani* kinesin-related protein, has been used successfully and showed high sensitivity and specificity. The rK39 dipstick showed a sensitivity of 95% (95 positive samples among 100 from VL patients) .

The total human patients involved in this study were 100 divided into four age groups . Kala-azar infection found among children under one year old of the patient groups 60 (60 %).This higher rate than the other age groups which was began to decrease reaching to(1%) in age more than 40 years of age groups . Males were high infected 62 % (62/100) than females 38% (38/100)in patient groups Therefore, the ratio of male / female was 1.6 / 1.

1. Introduction :-

Protozoan parasites of the genus *Leishmania* are widely distributed and transmitted by the bite of sandflies. In the vertebrate host, the infecting pro-mastigotes differentiate into and replicate as amastigotes within macro-phages. Symptoms range from self-healing skin lesions to diffuse cutaneous andmucosal manifestations, or severe visceral involvement of the spleen, liver, and lymph nodes. Visceral leishmaniasis (VL) is generally caused by *Leishmania donovani* in Africa, Asia, and southern Europe or *Leishmania chagasi* in Latin America. In VL, high antibody levels are observed prior to
the detection of parasite-specific T-cell responses (Ghose *et al*., 1980). This antibody response has been exploited for the diagnosis of *L. chagasi* and *L. donovani* infection (2-5). The current World Health Organization's estimate of 12 million cases of leishmaniasis and recent epidemics of VL in Sudan and India (WHO, 1991) highlight the need for more effective control measures. Diagnostic tests use whole or lysed *Leishmania*, and a few studies have begun to examine patient antibody responses to specific antigens (Reed *et al*.,1990).Such studies may improve diagnostic assays and help to evaluate B-cell responses during disease progression.

A variety of immunologic methods have been used to diagnose VL. Among others, the rK39 dipstick test is used because of its ease in handling, quick results, and high sensitivity and specificity. However, the sensitivity varied considerably in different disease-endemic areas. In India and Nepal (Sundar *et al*., 1998 ; Bern *et al*., 2000) the test showed the highest sensitivity (100%), but the sensitivity was significantly lower in Venezuela (88%) (Delgado *et al*., 2001) southern Europe (71.4%)(Jelineck *et al*., 1999) and Sudan (67%)(Zijlstra *et al*., 2001). This variation may be due to differences in the test accuracy between subspecies of *L. donovani* complexes, genetic differences in individual patients or in racial subgroups, and epidemiologic factors such as length or severity of diseases(Carvalho *et al*., 2003). Some persons with VL do not show any clinical manifestations. Khalil and others (Khalil *et al*., 2002) reported that in eastern Sudan the ratios of clinical and subclinical cases in 1994–1995 and 1995–1996 in Um-Salala village were 1.2:1 and 2.6:1, respectively, and in Mashrau Koka village were 1:11 and 1:2.5, respectively. In another study conducted in Bihar State, India, 69% of asymptomatic seropositve cases detected by the rK39 ELISA and dipstick test developed kala-azar within one year(Singh *et al*., 2002) which suggested that that many of the asymptomatic cases were in a pre-clinical state. Such early diagnosis will have a practical importance now that oral treatment with miltefosine has become available(Jha *et al*., 1999). Measurements of *Leishmania*-specific IgG, IgM, IgE, and IgG subclasses were also found to be useful as markers for active VL cases and for monitoring effective treatment (Anam *et al*., 1999 ; Atta *et al*., 1998).

One approach to diagnosis of visceral leishmaniasis (VL) (kala-azar) involves an immunochromatographic strip test that detects immunoglobulin G (IgG) antibody to recombinant K39, an antigen expressed by leishmanial species that produce VL (Boelaert *et al*., 2004 ; Carvalho *et al*., 2003 ; Veeken *et al*., 2003). A promising ready-to-use immunochromatographic strip test based on rk39 antigen has been developed as a rapid test for use in difficult field conditions. In this test, either serum or blood is smeared at the tip of the strip and anti k-39 antibodies leads to development of a red colour band 1 cm below a similar control band .This test is read with naked eye and the strips can be stored between 25-40°C for at least a year. The test has a sensitivity of 100 % and specificity of 98% (Bern *et al*., 2000).

2. Materials and Methods :-

The rK39 antigen-based dipstick test (InBios International, USA) was carried out according to the manufacturer's instruction. Briefly, 20 µL of serum was added to a test strip and two drops of chase buffer solution were added. The test result was read within 10 minutes after addition of serum. Even a weak line was considered positive. One hundred serum samples from defined VL patients collected from different areas of Wasit province in Iraq were used to compute sensitivity of the rk39 dipstick test.

Appearance of a purple upper control line indicated the presence of IgG and correct strip test functioning; a second lower purple line indicated the presence of anti-K39 IgG. The test strip membrane is coated with a band of recombinant K39 antigen and above the band with immobilized anti-protein A antibody to detect IgG; protein A-gold conjugate is used as the detection reagent. Anti-K39 IgG reacts with the protein A-gold conjugate and the mixture moves up the strip by capillary action to react with the K39 antigen, giving rise to a colored band in the test area. The rk39 dipstick test consists of individually packaged strips and a separate bottle containing buffer. According to the manufacturer, this test is not intended for use with whole blood; therefore, only serum was tested by the rk39 dipstick test. After 10 min, two pink bands indicated the presence of anti-K39 IgG and a positive result (Sundar *et al*., 2002).

3. Results and Discussion :-

3.1 Results :

3.1.1 Dignosis of Kala-azar by Serum rK39 Dipstick

Out of 100 patients, 95(95%) were found to be positive by serum rK39 dipstick test and 5 (5 %) were negative (Table 3.10), giving a sensitivity for rK39 dipstick of 95 % and specificity of 100 % (Table 3.1.1).

Table (3.1.1) Distribution of Kala-azar patients

		Kala azar patients	**%**
		Wasit province	
Serum rK-39 dipstick	**Positive**	**95**	**95%**
	Negative	**5**	**5%**
Total		**100**	**100%**

3.1.2 Distribution of Kala-azar Patients According to the Age

The total human patients involved in this study was divided into four age groups (Table and figure 3.1.2). Kala-azar infection found among children under one year old of the patient groups 60 (60 %).This higher rate than the other age groups which was began to decrease reaching to(1%) in age more than 40 years of age groups (Table 3.1.2).

Table (3.1.2) Distribution and percentage of Kala-azar according to the age

Age groups / Year		Studied groups		Total
		Kala- azar Positive	Kala-azar Negative	
G1(< 1)	N	60	2	62
	%	60	2	62 %
G2=1-20	N	21	1	22
	%	21	1	22 %
G3=21-40	N	13	1	14
	%	13	1	14%
G4(> 40)	N	1	1	2
	%	1	1	2%
Total	N	95	5	100
	%	95 %	5 %	100 %

3.1.3 Prevalence of Kala-azar in Relation to Gender

Males were high infected 62 % (62/100) than females 38 % (38/100) in patient groups(Table 3.1.3). Therefore, the ratio of male/female was 1.6 / 1.

Table (3.1.3) Gender distribution of Kala-azar

Age groups / Year		Gender		Total
		Male	Female	
< 1	No.	38	24	62
	%	34 %	28 %	62 %
1-20	No.	14	8	22
	%	14 %	8 %	22 %
21- 40	No.	8	6	14
	%	8 %	6 %	14 %
> 40	No.	2	0	2
	%	2 %	0 %	2 %
Total	N	62	38	100
	%	62 %	38 %	100.0%

P-value	C.S
	Non
0.596	Sig.

3.2 Discussion :-

Infection with *L. donovani* in the Iraq may cause a variety of clinical conditions that differ in morbidity and management. Differentiation between these conditions is important for our understanding of the epidemiology and the development of control strategies. Serological tests may be useful for differentiating between these clinical conditions. Unlike the antigens in currently used serological tests, rK39 antigen is specific for members of the *L. donovani* complex (Burns et al., 1993), and, as it occurs predominantly on amastigotes, it reflects infection by *L. donovani* followed by active parasite replication. In the diagnosis of kala-azar, rK39 dipstick showed higher sensitivity. The rK39 titers remain positive for up to 2 years after treatment. Thus, rK39 dipstick does not allow discrimination between active infection and past infection.

A similar capability to detect infection earlier was shown for subjects with subclinical infection who were diagnosed by seroconversion. Almost most of these individuals had already converted in rK39 dipstick (6 months before), showing that rK39 dipstick is superior in detecting infection at an earlier stage. Our findings are consistent with prior observations from researchers using this test in Venezuela (Delgado et al., 2001), and in contrast to a study in Brazil in which antibodies to rK39 were absent in children who had subclinical disease and self-healed without specific therapy (Badaró et al., 1996**).** Our findings indicate that K39-positive individuals may self-heal or proceed to clinical disease. Several studies have been carried out to assess sensitivity and specificity of this test, and especially how it compares to other methods. Sundar *et al* and Goswami *et al* found 100% sensitivity and 98 %specificity in 2 separate and independent studies.

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