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Evaluation of the Efficacy of Different Serological Methods (rK39 dipstick, ELISA and IFA) Techniques in the Diagnosis of Visceral leishmaniasis.

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Abstract

The objective of this study was to compare the sensitivity and specificity of rK39, ELISA and IFA tests for the diagnosis of visceral leishmaniasis using blood samples. Samples were collected from 146 suspected patients and 30 healthy individual as control. The results showed 52 (35.62%), 58 (39.73%) and 71 (48.64%) positive samples by rK39, ELISA and IFA , respectively. IFA test showed a higher sensitivity (48.63%) than ELISA and rk39 dip stick (39.72%) and (35.61%) respectively. The highest sensitivity of agreement (K: 0.912) was found between rK39 dipstick and ELISA. Therefore, we suggest the application of rK39 dipstick and ELISA methods in endemic areas of visceral leishmaniasis for early and accurate diagnosis of this disease.

Keywords: Visceral leishmaniasis, rK39 Dipstick, ELISA, IFA.

تقييم كفاءة طرق مصلية مختلفة (دب ستك، ايلايزا،ايفا) في تشخيص الاصابة باللشمانية الاحشائية

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الخلاصة

تهدف هذة الدراسة مقارنة الحساسية والنوعية لثلاث طرق مصلية (دب ستك ،ايلايزا وايفا) من اجل تشخيص الاصابة باللشمانيا الاحشائية .تم جمع عينات الدم من 146 شخص مشكوك باصابتهم بهذا المرض و30 شخص اصحاء كمجموعة مقارنة. اظهرت النتائج ,(35.62%)52 و (39.73%)58 و (48.64%)71 في الاختبارات الثلاث (دب ستك ايلايزا وايفا) على التعاقب.اختبار ايفا اظهر اعلى حساسية (48.63%) من دب ستك والالايزا (35.61%) (39.72%) على التعاقب. تم حساب قيمة كابا بين كل اختبارين من هذة الاختبارات الثلاثة بين دب ستك وايلايزا،دب ستك وايفا وايلايزا وايفا، اعلى قيمة كابا كانت بين دب ستك وايلايزا.استنتج من هذة الدراسة ان الدب ستك مع الاليزا هي افضل الطرق المصلية لتشخيص الاصابة باللشمانيا الاحشائية.

Introduction

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania* [1]. Female sand fly of genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World are the only proven vector responsible for transmission of the disease [2].

There are three major forms of leishmaniasis in human cutaneous leishmaniasis (CL) mucocutaneous (MCL) and visceral leishmaniasis (VL). Visceral leishmaniasis (VL) is one type of

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leishmaniasis infections that remains asymptomatic or subclinical in many cases or can follow an acute or chronic course. The clinical symptoms are characterized by prolonged and irregular fever often associated with rigor and chills, splenomegaly lymphadenopathy, hepatomegaly, pancytopenia progressive anaemia and weight loss. It is always fatal if left untreated [3].

Infection of leishmaniasis is also characterized by the appearance of anti leishmanial antibodies in the sera of patients. In the CL, usually they are present at low levels during the active phase of the disease. In contrast strong anti-leishmanial antibody titters are well documented in VL Critical analysis of *Leishmania* antigen-specific Immunoglobulin (Ig) isotype revealed elevated levels of IgG, IgM and IgE during disease [4].

Diagnosis of VL generally relies on microscopic examination, mostly based on bone marrow aspirate, and culture in (NNN) medium. In addition, specific serological tests such as indirect immunofluorescent antibody technique (IFAT), enzyme-linked immunosorbent assay (ELISA) and rK39 dipstick technique [5].

Several serological tests including rK39 antigen dipstick (r K39), enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFA) have provided good efficacy in diagnosis of VL [6]. The sensitivity and specificity of such diagnostic methods depends on the type, source and purity of antigens employed, as some of *Leishmania* antigens have common cross-reactive epitopes shared with other microorganisms [7].

RK39 antigen dipstick test developed against a recombinant antigen derived from 39 amino acid repeat in *Leishmania chagasi* (rK39). It is of high sensitivity when used as an enzyme linked immunosorbent assay and was developed in a simpler dipstick format.

Evaluation of rapid diagnostic tests [8].

IFA and ELISAs are useful diagnostic tools. In general, the assays that use whole parasite antigens have high sensitivity but relatively low specificity because of cross-reaction with Chagas disease, malaria and other infections (as well as non-specific cross-reactivity).

Materials and Methods

The study was carried out from September 2010 to April 2011on patients attended the central heath laboratory in Baghdad ,5 ml venous blood samples were collected from each patient suspected with VL and serum were separated to submit the three serological tests:

rK39 dipstick

The principle of this technique is qualitative and performed according to (InBios International Seattle, USA). membrane based immunoassay for the detection of antibodies to VL in human serum. The membrane is pre-coated with rk39 on the test line region and chicken anti- protein A on the control line region. During testing, the serum sample reacts with the dye conjugate (protein A-colloidal gold conjugate) which has been pre-coated in the test device. The mixture then migrates up ward on the membrane chromatographically by capillary action to react with recombinant VL antigen on the membrane and generates a red line.

Presence of the red line indicates a positive result, while its absence indicates a negative result. Regardless of the membrane to the immobilized chicken anti-protein a region, a red line at the control line region will always appear. The presence of this red line serves as verification for sufficient sample volume and proper flow and as a control for the reagents.

ELISA technique

The test was performed according to the manufacturer's instruction of (Vircell microbiologists, Granada, Spain). ELISA techniqueis based on the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Unbound immunoglobulins are washed off. An enzyme-labeled anti-human globulin binds the antigen-antibody complex in a second step. After a new washing step, bound conjugate is developed with the aid of a substrate tetramethyl benzidine (TMB) to render a blue colored soluble product which turns into yellow after adding the acid stopping solution. Finally the optical density was measured at 450/620 nm. Samples with indexes below 9 are considered as not having antibodies against *Leishmania.* Samples with indexes above 11 are considered as having antibodies against *Leishmania.* Antibody index= (sample O.D. /cut off serum mean O.D.)X10.

IFA technique

The IFA technique is based upon the reaction of antibodies in the sample, react with the antigen adsorbed on the slid surface. The specific antibodies present in the sample react with the antigen, and the immunoglobulins not binded to the antigen are removed in the washing step. In the next step, the

antigen-antibody complexes react with the fluorescein-labeled anti-human globulin. It can be examined using an immunofluorescence microscope. (Vircell microbiologists,Granada, Spain).

Statistical analysis

Sensitivity was estimated by the classic validation method. The degree of agreement between rK39 dipstick, ELISA and IFA tests was determined by calculating Kappa values with 95% confidence intervals using Epi-info. Kappa values express the agreement beyond change, and a value of 0.21–0.60 represents a fair to moderate agreement, 0.60–0.80 a substantial agreement and >0.80 almost perfect agreement beyond change. Using Chi-Square test and a P-value < 0.05 was considered statistically significant.

Results and Discussion

This study showed that out of 146 suspected patients, 52 (35.62%) patients revealed positive results for VL by rK39 dip stick test (25 male and 27 female), 58 (39.73%) patients scored positive results by ELISA (26 male and 32 female) and 71 (48.64%) patients gave positive results by IFA test (35 male and 36 female) there was no significant differences between the three tests at *P* value < 0.05 (Table1) Out of 146 samples 46 are less than one year, 5 are 6-10 years IFA test showed a higher sensitivity (48.63%) than ELISA and rk39 dip stick (39.72%) (35.61%) respectively (Table 2), this result agreed with that obtained by [9] who found that rK39 dipstick showed the lowest sensitivity in comparison with PCR and ELISA. Both rK39 dipstick and IFA tests showed sensitivity and a specificity of and specificity of (89.65%) (100%) respectively. Using IFA with ELISA showed a sensitivity of (81.69%) and a specificity of (100%). Statistical analysis of the data showed a substantial agreement (kappa=0.73) between dipstick and IFA whereas a perfect agreement (kappa=0.820) was found between IFA and ELISA. The best results were observed between ELISA and dipstick (kappa=0.912) (Table 3, 4 and 5). Various serological tests, with different specificities and sensitivities, are available for the diagnosis of leishmaniasis. In this study, the results showed that the rK39 dipstick and ELISA techniques are equally accurate for the detection of anti-*Leishmania* antibodies; the sensitivity was 100% for rK39 dipstick and was 89.65 % for ELISA, while [10] found that the rK39 dipstick and IFA instead of ELISA are the best techniques for the diagnosis of VL. The results of this study confirmed those reported by [11-15] in which the rK39 dipstick test was used for the diagnosis of human visceral leishmaniasis, with a specificity of 97%.

In the last years, an increasing number of *Leishmania* antigens have been employed in ELISA systems for diagnosis of VL. Some of which are considered to be species specific proteins such as gp46, lipophosphoglycan-associated protein KMP11, kinesin, heat shock protein, actin and tubulin [16]. Kau, *et al*.,[17] evaluated a 200 KDa antigen fraction from *L. donovani* axenic amastigotes and also *L. donovani* soluble antigen in ELISA system for the detection of antibody in VL patients. A positive antibody response in confirmed VL cases was seen in 96.6% of cases, when using the 200 KDa fractions and 100% when the amastigote soluble antigens were used. In our study ELISA with dipstick was the most sensitive method for diagnosis of VL. IFA test is a commonly used technique for Kala azar. This is a sensitive test requires low amount of antigens and detects the low titer of antibodies present in patient serum, which are demonstrated in the very early stage of infection and are undetectable six to ninth months after cure [18] Based on our study, IFA test will be our method of choice for implementation of a serological test for the diagnosis of VL when it is used separately from other tests, but the use of rK39 dipstick with ELISA is proper for decisive diagnosis of the

Table 1- Comparison of the performances of rK39, ELISA and IFA for the diagnosis of VL.

Type of technique	Patients		
	Positive	Negative	Total
RK39	52 (35.62%)	94(64.38%)	146(100%)
ELISA	58(39.73%)	88(60.27%)	146(100%)
IFA	71(48.64%)	75(51.36%)	146(100%)

Table 2- Comparison of the sensitivity of rK39, ELISA and IFA techniques.

Technique	rK39	ELISA	IFA
Sensitivity	35.61%	39.72%	48.63%

Table 3- Sensitivity and specificity of dipstick and IFA techniques.

Technique	Sensitivity %	Specificity %
Dip Stick	100	79.78723
IFA	73.23944	100
Kappa value: 0.737		

Table 4- Sensitivity and specificity of ELISA and dipstick.

Technique	Sensitivity %	Specificity %
Dip stick	100	93.61702
ELISA	89.65517	100
Kappa value: 0.912		

Table 5- Sensitivity and specificity of IFA and ELISA technique.

Technique	Sensitivity%	Specificity%
ELISA	100	85.22727
IFA	81.69014	100
Kappa value: 0.820		

Disease. This is especially important in endemic areas of the disease since rK39 dipstick test is ideal for use under field conditions because it can be read visually and does not require laboratory equipment, electricity, or refrigeration. Moreover, whole blood can be collected, placed directly in the well, and tested at a later stage.

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