Performance of Recombinant rK39 antigen in the diagnosis of Leishmaniasis

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Abstract

Background: Recombinant K39 antigen test is one of the recent experiences in rapid diagnosis of Visceral Leishmaniasis ,it consist of 39 amino acid which represent the main component of the protein of the Leishmania parasite and that the reaction occurs between the antibodies in the serum and antigens blotted in the rK39 strip test.

Objective: Find out possible reaction of rK39 antigen test on other diseases than visceral leishmaniasis, as well as to compare the test results on both serum and urine of same patients.

Patients and Methods: A total of 65 blood and urine samples were included in the study; 25 patients with cutaneous leishmaniasis attended Baquba Teaching Hospital, and 40 apparently healthy controls. All serum and urine samples were tested with rK39 strip test. 15 serum samples were randomly selected and Indirect Fluorescent Antibody Techniques for diagnosis of Leishmaniasis for the purpose of comparison of rK39 strip test**.**

Results: There was highly significant differences when using the rK39 strip test in urine and serum in patients infected with cutaneous leishmaniasis ($P < 0.05$), and no significant difference between the sexes, as well as age group , as well as the absence of a significant difference between the rK39 strip test and Indirect Fluorescent Antibody Techniques.

Conclusion: rK39 strip antigen test gives positive results, may be due to greater phylogenetic proximity between Leishmania species, or due to mixed infection with Visceral Leishmaniasis.

Key words: rK39 antigen test, leishmaniasis, IFAT.

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Introduction

Leishmaniasis is a disease resulting from infection with one of 20 species of the protozoan parasite. *Leishmania* is transmitted by Phlebotomies sand fly species [1]. Occasional non vector transmissions have also been reported through blood transfusions, sexual intercourse, organ transplants, excrements of dogs, and sporadically outside endemic areas [2].

Clinically, leishmaniasis manifests itself in four main forms: cutaneous, mucocutaneous, diffuse cutaneous and visceral [3]. Leishmaniasis is endemic in 88 countries spread over five continents, with an estimated yearly incidence of 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis (VL). The number of people at risk is estimated to be 350 million, with an overall prevalence among 12 million people [4]. Recombinant K39 antigen test is one of the recent experiences and rapid diagnosis of

visceral leishmaniasis using antigen rK39 ,it consist of 39 amino acid which represent the main protein of the parasite and that the reaction occurs between the antibodies in the patient serum and antigens installed to the rK39 strip test [5]. And that this test has a high sensitivity and specificity in South Asia, but less sensitive in East Africa, which may be one of the reasons behind the difference in specific continent due to the molecular diversity of antigen rK39 within *L. donovani* complex however may be a second reason for the difference in the levels of lgG antibodies against Leishmania parasite in patients suffering from kala azar and who belong to different geographical regions either because of antigenic changes or immune response [6].

So the aims of the study is to find out possible reaction of rK39 antigen test on other diseases than visceral leishmaniasis, as well as to compare the test results on both serum and urine of same patients.

Patients and Methods

This study was conducted in Diyala province, the period between 7/ 1/ 2015 to 23/11/2015, which aims to evaluate the rK39 strip test in the diagnosis of leishmaniasis, where execution questionnaire includes several variables for each individual, include (sex, age). A total of 65 blood and urine samples were included in the study; 25 patients with cutaneous leishmaniasis attended Baqubah Teaching Hospital and 40 apparently healthy controls. Blood samples were aspirated by sterile syringe in gel tube and then serum was separated by centrifugation as well as the urine samples were collected by special glasses. All serum and urine samples were examined by rK39 strip testing (Bios, USA) and then selected a 15 sample of serum patients with cutaneous leishmaniasis were examined by Indirect Fluorescent Antibody Techniques(IFAT)(Euroimmun medizinische labordiagnostika AG German) For the purpose of comparison rK39 strip test.

rK39 (Dipstick): Assay procedure

The Kala-azar dipstick strip was removed from the pouch or vial. one drop of serum and urine was placed on the strip bottom, the test strip is placed in to test tube so that the end of the strip faced down word, the mixture is allow to migrate up to the strip by capillary action 2-3 drops were added of the buffer solution provided with the test kit to the pad, the results recorded in 10 minutes. The test was positive when a control line and test line appear in the test area with color red. The test is negative when only the control line appears. The test is invalid if no control line appears regardless of whether the test line is seen.

IFAT test: Assay procedure according to the manufactures' instructions as follows:-

Preparation of the reagents and of the serum. We applied 30µl of diluted serum to each reaction field of the reagent tray avoiding air bubbles all amples to be tested were transferred before starting the incubation by use a polystyrene pipetting, reactions started by fitting the biochip slides into the corresponding recesses of the reagent tray ensure that each sample makes contact with its biochip and that the individual samples do not come into contact with each other, incubated for 30min at room temperature (+18C to +25C), the biochip slides was rinsed with a flush of PBS-Tween using a beaker and immersed them immediately afterwards in a cuvette containing PBS-Tween for at least 5min, shacked with a rotary shaker , washed max 16 slides then replaced with PBS-Tween with new buffer. 25ml of fluorescein labeled anti-human globulin applied to each reaction field of a clean reagent tray, all droplets was added before continuing incubation, a stepper pipette the labeled anti-human serum was mixed before use to save time, conjugate can be pipetted onto separate reagent trays during the incubation with the diluted sample.

One biochip slide removed from cuvette within five seconds, only the back and the

long sides was blotted with a paper towel and immediately , the biochip slide inserted into the recesses of the reagent tray , then continued with the next biochip slide, the slides was protected from direct sunlight, incubated for 30min. at room temperature (+18C to +25C). Cuvette filled with new PBS-Tween, the biochip slide rinsed with a flush of PBS-Tween using a beaker and inserted them into the cuvette filled with the new PBS-Tween for at least 5min, shacked with a rotary shaker. 10 drops of evans blue for each 150µl phosphate buffer can be added for counterstaining.

Embedding medium placed onto a cover glass drops per reaction field in a polystyrene embedding template, one biochip slide removed from PBS-Tween and dry , the biochip slide inserted with the biochips facing downwards onto the prepared cover glass check immediately that the cover glass is properly fitted into the recesses of the slide. The fluorescence recorded with the microscope. Fluorescence pattern (positive reaction) antibodies against leishmania result in a smooth either complete or rimmed fluorescence of the cell body, positive reaction cells mast be clearly identifiable in every field examined preferably in several areas fluorescence of only some of the cells or exclusively the cell

Statistical analysis

Date entered into a computer using the statistical package for social science (SPSS) version [18]. Quantitative variables summarized by finding mean ±SD. P-value of < 0.05 consider as statistically significant by using Chi Square test.

Results

Cutaneous Leishmaniasis.

Comparison of urine and serum infected with cutaneous leishmaniasis rK39 strip test using

The Results showed to analysis conducted on 25 samples from patients infected with cutaneous leishmaniasis and the presence of a highly significant difference between urine and serum where situation rK39 strip test, total number of positive samples in the case of urine [20] (80%), while the number of positive samples of serum were [5] (20%), as shown in the table [1].

Table (1): Percentage of positive and negative results of urine and serum samples of patients with cutaneous leishmaniasis using rK39 strip test.

Test type	Number	positive		negative	
rK39		N	%	N	%
Urine	25	20	80	5	20
Serum	25	5	20	20	80

Chi-Sq=9.000, **df**=1, **P**<0.05, **Sig**.=0.003

Polymerase as for the effect of sex results of statistical analysis, no significant difference between males and females using the rK39 strip test in urine, reaching the number of positive samples in urine for males [6] sample and that percentage was (66.66%), while the females were a number of positive samples for urine [14] samples and the percentage was (87.5%), as in the table [2].

Table (2): Percentage of positive and negative results of urine males and females using the test rK39 strip

Test type	Number	Positive		Negative	
rK39		N	%	N	%
Males	9	6	66.66	3	33.33
Females	16	14	87.5	2	12.5
Total	25	20		5	

Chi-Sq=3.200, **df**=1, **P**>0.05, **Non-Sig**=0.074.

The results showed statistical analysis for the age group the rK39 strip test using urine also no significant difference between individuals less than 15 years and greater than 15 years, and the number of positive samples for individuals less than 15 years of [9] sample and the percentage (81.81%) and reached number positive samples greater than 15 years for individuals [11] and that the sample percentage was (78.57%), as shown in the table [3].

Table (3): Percentage of positive and negative results between age groups using rK39 strip test in urine.

Test type	Number	positive		negative	
rK39		**N**	**%**	**N**	**%**
Less than 15 years	11	9	81.81	2	18.18
Greater than 15 years	14	11	78.75	3	21.42
Total	25	20		5	

Chi-Sq=0.200, **df**=1, **P**>0.05, **Non Sig**=0.655

Comparison between rK39 strips test with IFAT test for patients infected with cutaneous leishmaniasis.

Fifteen samples were examined randomly from patients with cutaneous leishmaniasis (Baghdad boil) using the indirect fluorescence antibody test for serum and then were compared with the rK39 strip test for the same serum as well as showed the results of statistical analysis, no significant difference between the two tests (P> 0.05) reaching the number of samples positive for serum of individuals diagnosed rK39 strip testing [4] sample and that percentage was (26.66%), while in the IFAT test case amounted to a number of positive samples as in rK39 strip test [4] sample and its percentage was (26.66 %), as shown in the table (4).

Table (4): Results of serum samples from patients infected with cutaneous leishmaniasis using rK39 strip test and IFAT test.

Test type	Number	Positive		Negative	
		N	**%**	**N**	**%**
rK39 strip test	15	4	26.66	11	73.33
IFAT test	15	4	26.66	11	73.33

Chi-Sq=0.00, **df**=1, **P**>0.05**, Non-Sig**=1.000

Healthy control

Forty healthy individuals that are considered as a control group were examined using rK39 strip test in urine and serum, the number of positive samples for urine was [4],there percentage was (10%),the serum did not record any positive cases, as shown in table(6).

Table (5): Results of urine and serum samples in healthy control individuals using rK39 strip test.

Test type	Number	Positive		Negative	
		N	%	N	%
Urine	40	4	10	36	90
Serum	40	0	0	40	100

Discussion

This study included 25 patients with cutaneous leishmaniasis, their urine and serum samples were checked with rK39 strip test and also 15 random serum samples were examined by IFAT test specific for Visceral Leishmaniasis for the purpose of comparison with the test rK39 serum.

The results showed highly significant difference (P <0.05) between the urine and serum in case rK39 strip test and the number of positive samples for urine and serum are 20 (80%) and 5 (20%), respectively, other study on the rK39strips test using serums, showed that a small percentage of patients who suffer from various diseases such as tuberculosis and malaria, gave a false positive result. this results differ slightly with this study [7], which included six cases of post kalazar dermal leishmaniasis (PKDL) and 3 patients with visceral leishmaniasis and human immunodeficiency virus (HIV) combined infection where showed the result is positive with both serum and urine (100%), this study completely identical with present study and the reason for our frequent false positive results, especially in patients of cutaneous leishmaniasis and specifically with the urine may be due to the insect vectors possibly infected with L.donovani that is the infection is possible to be mixed or may be due to greater phylogenetic proximity between *Leishmania* species . Other studies have confirmed that high-sensitivity assay using rK39 for the diagnosis of visceral leishmaniasis disease and (PKDL) and most encouraging reports are mostly from the Indian subcontinent, reaching sensitivity (89-100%) (8 : 9 : 10: 11 : 12 (.Isalm et al ., (2004) explained infected with visceral leishmaniasis and 137 infected with various diseases (malaria, tuberculosis, aplastic anemia, nephrotic syndrome), as well as healthy and using screening DAT based on urine (antibodies lgG) results showed the presence of a false positive result for each of the different diseases and when adding enhancing antibodies for (75 infected with visceral leishmaniasis and 225 of those with various illnesses) led to a significant improvement in test DAT-based urine where 68 of the total 75 positive samples in the urine individuals infected with visceral leishmaniasis and 217 of the total 225 sample gave a negative result [13]. In another study showed that the five positive samples out of 25 samples from malaria patients positive results with rk39 [14]. This is consistent with

studies in the Indian subcontinent and East Africa and Brazil (15: 16). As well as in the Indian study published recently been some cross-reactions with the statement that exist control where the disease is endemic and people who have other diseases [17].

As for the effect of gender and age on the diagnosis using test rK39 strip test in patients with cutaneous leishmaniasis. The results showed non-significant difference (P> 0.05) between the male and female gender, as well as ages less than 15 years and greater than 15 year. Despite the scarcity and lack of studies on the effect of age and sex in the diagnosis of various diseases using test rK39 in patients infected with cutaneous leishmaniasis, but there are studies close to that reported [18]. This is in agreement with other studies in Iraq carried others; [19, 20, 21, 22]. These studies recorded that males were more affected than females, and the reason may be because females are more protective covering all her body clothes and less vulnerable to insect vector.

Also 15 serum samples in this group were compared the rK39 strip test with IFAT test randomly from patients with cutaneous leishmaniasis which showed non-significant difference (P> 0.05) between the two tests, where the percentage of the number of positive samples for both tests performed (26.66%), but these results may be false positive because of the similarity of the factors causing or that Sand fly vector carrying or infected with parasites that cause cutaneous and visceral leishmaniasis so it may be a combined infection with predominance of the infection on the skin only, other possible cause may be due to cross reactive antigen. Al-Janabi *et al* ., (2014) in another study in the Maternity and Children's Teaching Hospital in Aldewanh which included 687 patients to evaluate the rK39 strip test on patients black fever validity in order to detect antibodies in human blood serum, where the results were positive in all patients who are diagnosed with a black fever by IFAT test [23].

In this study, a group of 40 individuals from healthy individuals that are considered as a control group we examined their urine and serum using rK39 strip test showed the number of samples positive and negative for the urine of 4 (10%) 36 (90%) respectively. As for the serum did not record any positive cases and all of them gave a negative result of 40 (100%). Singh et al., (2009), included 210 cases were 100 cases of infection visceral leishmaniasis been confirmed and 50 cases of healthy individuals in endemic areas and 20 cases of healthy people in non-endemic areas and 40 cases of people infected with febrile diseases different. All groups were examined with rK39 strip test, showed that 98 positive cases of the total 100 cases tested for the visceral leishmaniasis and 5 of the total 50 healthy individuals in endemic areas give positive results [24].

The disease has not been found on any healthy individuals in non-endemic areas of the disease, as well as individuals with febrile illnesses, in addition to that it showed rK39 strip test positive results of 50 cases after the completion of successful treatment for a period of 180 days has up to six months and this is in line with the results of the current study, and the healthy (control) positive results in some cases may be due to these people have subclinical infection or they may be in the incubation period of the disease or may be subjected to individual pathogen slightly due to the immune system and overcome the disease [24]. As well as in another study Conducted of 150 cases where the proved infection visceral leishmaniasis disease examined by rK39 strip testing serum in terms of number of positive samples amounted to 148 (98%) as well as 160 individual healthy controls were examined in the same test as the number of positive samples of 17 (10.6%). Khan et al., (2010),

included 50 healthy individual in infected and non-infected areas have been examined the rK39 strip test using for the urine where didnt found any positive result , and this is different with the results of our study which found that the number of positive samples for the urine of 4 (10%) [14]. This in conformity with the present study, which did not distinguish between healthy controls in endemic areas and non- endemic. In a study conducted by Singh et al., (2013) on 421 healthy individuals were examined urine and serum using rK39 strip test where he not found any positive cases of healthy in non-endemic areas disease, while the percentage of samples positive for to the Healthy persons in endemic areas of (6.2%) [25]. This corresponds with the results of other studies in the Indian subcontinent and East Africa and Brazil (15 : 16).

In conclusion, rK39 strip antigen test positive results in patients with cutaneous leishmaniasis, it may be due to greater phylogenetic proximity between Leishmania species, or due to mixed infection with Visceral Leishmaniasis.

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