Correlation of Rk39-Specific Antibodies and Thyroid Function in Patients with Visceral Leishmaniasis

Hastalarda Rk39’a Spesifik Antikorların ve Tiroid Fonksiyonunun Visseral Leishmaniasis ile Korelasyonu

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ABSTRACT

Objective: This study aimed to determine whether anti-rK39 antibodies were diagnostic markers for visceral leishmaniasis (kala-azar) and to evaluate the correlation between age and gender in disease occurrence in Iraqi patients. In addition, it aimed to evaluate the correlation between thyroid hormones, i.e., thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) and anti-rK39 antibodies.

Materials and Methods: Immunochromatographic technique used for anti-rK39 antibodies detection. Enzyme-linked immunosorbent assay was used for determining the serum TSH, T3, and T4 levels.

Results: One hundred thirty-eight patients with visceral leishmaniasis were included. The mean age was 27.65±11.60 years. Sixty-one patients (44.2%) were males, and their mean age was 29.65±11.10 years. The mean age of females was 26.12±11.89 years. Anti-rK39 antibodies were detected in 11.59% of patients. Anti-rK39 antibodies were equally detected (5.8%) in both genders without a significant difference (p=0.212) or correlation between gender and anti-rK39 antibodies (p=0.623). There was neither a significant difference (p>0.05) nor correlation between gender; age groups according to gender and anti-rK39 antibodies (p>0.05). Both males and females who were positive for anti-rK39 antibodies had normal TSH, T3, and T4 levels. Only one patient who was positive for anti-rK39 antibodies had an elevated T4 level (>12 µg/dL). Neither a significant difference nor correlation was reported among genders; anti-rK39 antibody positivity (p>0.05); and TSH, T3, and T4 levels.

Conclusion: Anti-rK39 antibodies, a diagnostic marker for visceral leishmaniasis have no correlation with patients age and gender. Serum TSH and T3 levels were not affected by visceral leishmaniasis. Visceral leishmaniasis causes the increase in serum T4 levels. Thyroid involvement appears to be uncommon in patients who present with visceral leishmaniasis.

Keywords: rK39-specific antibodies, thyroid-stimulating hormone, triiodothyronine, thyroxin.

ÖZ

Amaç: Bu çalışmanın amacı anti-rK39 antikorlarının visseral leishmaniasis (kala-azar) için tanisal belirteçler olup olmadığını belirlemek ve İraklı hastalarda hastalığın ortaya çıkmasıyla yaş ve cinsiyet arasındaki ilişkiyi ele almak için immunokromatografik tekniğin kullanıldığı ve anti-rK39 antikor pozitif hastaların TSH, T3 ve T4 seviyelerinin normal olup olmadığını belirlemektedir.

Gereç ve Yöntem: Anti-rK39 antikorlarının tespiti için immunokromatografik teknik kullanıldı. Serum TSH, T3 ve T4 seviyelerini belirlemek için Eliza testinden yararlanıldı. Serum TSH, T3 ve T4 seviyelerini belirlemek için Eliza testinden yararlanıldı.

Bulgular: Visseral leishmaniasis hastalığı olan 138 hasta çalışmaya dahil edildi. Ortalamalı yaş 27.65±11.60 yıldır. Hastaların 61’i (%44.2) erkekti ve ortalamalı yaşları 29.65±11.10 yıldır. Kadın hastaların ortalamalı yaşları 26.12±11.89 yıldır. Hastaların %11,59’unda anti-rK39 antikorları tespit edildi. Anti-rK39 antikorları cinsiyet ve anti-rK39 antikorları arasında anlamlı bir fark (p=0,012) ve korelasyon olmaksızın her iki cinsiyette de eşit şekilde bulunmuş (p=0,623). Cinsiyet, cinsiyete göre yaş grupları ve anti-rK39 antikorları arasındaki anlamlı bir fark (p<0,05) ya da korelasyon bulunmadı (p>0,05). Anti-rK39 antikorları açısından pozitif olan erkek ve kadın hastalarında TSH, T3 ve T4 seviyeleri normaldı. Pozitif anti-rK39 antikora sahip sadece bir hasta yüksek T4 seviyesi tespit edildi (>12 µg/dL). Cinsiyet, anti-rK39 antikor pozitifliği (p<0,05) ve TSH, T3 ve T4 seviyeleri arasında anlamlı bir farklık ya da korelasyon bulunmadı.

Sonuç: Anti-rK39 antikorlarının yaş ve cinsiyet ile korelasyonu yoktur. Serum TSH ve T3 seviyelerinin visseral leishmaniasis etkeniyle korelasyonu bulunmadı, Visseral leishmaniasis serum T4 seviyelerinde artışa neden olmadı. Tiroit tutulumunun visseral leishmaniasis olan hastalarda yaşın olmadığı görülmuştur.

Anahtar Kelimeler: rK39-spesifik antikorlar, tiroit stimule edici hormon, triiodotironin, tiyrosin
Introduction

Visceral leishmaniasis (VL; kala-azar) is a slowly progressing indigenous disease that is caused by a protozoan parasite of the genus Leishmania (Leishmania donovani, L. infantum, and L. chagasi). Leishmaniasis is transmitted by the bite of an infected female phlebotomus sand fly [1]. The life cycle of Leishmania involves two forms: promastigotes, wherein Leishmania develops and lives extracellularly in the sandfly vector, and amastigote, wherein Leishmania multiplies intracellularly in the reticuloendothelial cells of the host [2]. Rodents, dogs, and foxes are the reservoirs of the infection. In endemic areas, man is the main source of infection [1].

In its mammalian host, Leishmania survives in the severe environment of the phagolysosome and evades the defense mechanisms that are induced during the immune response. Patients with VL, particularly children and young adults, present with dark skin and brittle and dry hair of varying color tones on their head [3]. Histopathological studies have demonstrated the parasitism in endocrine glands, particularly the pituitary, adrenal, thyroid, and sex glands [4]. Pubertal retardation among these patients is frequent in both sexes, a complication of long-term progression of the disease in young people [4]. Data reveal the necessity for investigating the main hormonal alterations in patients with VL.

The diagnosis of Kala-azar usually depends on clinical features of the disease in an endemic area, which confirmed by either demonstration of the parasite in the splenic aspirate or indirect tests. The rk39 test kit is currently widely used [1]. Such a progressive infection is associated with poor delayed-type hypersensitivity and high antibody production [5]. While the gold standard for diagnosis remains the demonstration of parasites in splenic or bone marrow smears, serological tests such as the direct agglutination test and rk39-based tests, e.g., immunochromatographic strip tests or enzyme-linked immunosorbent assay (ELISA), are increasingly used for diagnosis [6, 7].

Because of the conditions that prevail in areas of endemicity like poverty, armed conflicts and lack of infrastructure and inaccessibility, sophisticated methods 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. 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 [8]. The rk39 immunochromatographic test gives reliable results of 100% sensitivity and 98% specificity when combined with a strict clinical case definition [9].

Although alterations of thyroid hormone levels because of non-thyroid illness are well known among humans, there were very few studies about the pathological changes in thyroid hormone levels among patients with VL. Under experimental infections with VL in canine, serum levels of TNFα and interleukin-6 were increased which contribute to the inhibition of hypothalamus–hypophysis–thyroid axis, resulting in a decrease in production, secretion, and circulation of thyroid hormones [10, 11].

This study aimed to determine whether anti-rk39 antibodies were diagnostic markers for VL (kala-azar) in Iraqi patients with clinical manifestation of the disease and evaluate the possible correlation between age and gender in disease occurrence. Furthermore, it aimed to evaluate serum levels of thyroid hormones (TSH, T3, and T4) and their possible correlation with anti-rk39 antibodies.

Materials and Methods

Selection of patients:

In this cross-sectional study, 138 patients who presented suspected clinical manifestations of kala-azar and who visited private and public out-patient clinics in Baghdad from June 2014 to May 2015 were selectively enrolled. This study was conducted according to the principles of the Helsinki declaration. Duly filled consent forms were obtained from all patients participating in the study. Approval of the Ethical Review Committee of the College of Medicine, Diyala University, Iraq was obtained before initiating the study.

The mean age of the patients was 27.65±11.60 years; the minimum age was 5 years and maximum age was 70 years. Sixty-one patients (44.2%) were males; their mean age was 29.65±11.10 years, minimum 5 years, maximum 56 years. The rest of the patients were females, with a mean age of 26.12±11.89 years, minimum age was (5) years and maximum age was (70) years.

Methods:

Five milliliters venous blood was aseptically obtained from all patients who presented with clinical manifestations of kala-azar. The samples were centrifuged at 2000 × g at room temperature for 5 min to separate sera. Separated sera were stored at −20°C until used for immunochromatography and ELISA.

Detection of anti-rk39 antibodies:

The detection of kala-azar (VL) depends on the immunochromatographic detection system of rk39-specific antibodies (Kalazar Detect™ Test), which is a qualitative membrane-based immunoassay for detecting rk39-specific antibodies to VL in the human serum [12]. The membrane is pre-coated with rk39 on the test line region and chicken anti-protein A on the control line region. During the test, 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 chromatographically migrates upward on the membrane by capillary action to react with the recombinant rk39 antigen on the membrane and generates a red line. The presence of a red line indicates a positive result, whereas its absence indicates a negative result. Regardless of the presence of antibody against rk39, as the mixture continues to migrate across 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 verifies the sufficient sample volume and proper flow and acts as a control for the reagent.

Thyroid function tests:

1. Thyroid-stimulating hormone (TSH)

In this study, the DRG TSH ELISA Kit, a solid phase ELISA based on the sandwich principle, was used to determine serum TSH levels according to the manufacturer’s instruction [13].

2. Triiodothyronine (T3)

The DRG Total T3 ELISA Kit, a solid phase ELISA [13] based on the principle of competitive binding, was used to determine serum T3 levels according to the manufacturer’s instruction.

3. Thyroxine (T4)

The DRG Total T4 ELISA Kit, a solid phase ELISA based on the principle of competitive binding, was used to determine serum T4 levels according to the manufacturer’s instruction [13].

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences for Windows version 17 (SPSS, Armonk, NY: IBM Corp.) and Microsoft Excel for Windows 2010. Pearson’s chi-square and Pearson’s correlation coefficient were used for correlation between the variables of the two tests. P value of ≤ 0.05 and ≤ 0.01 (two tailed) was set to be statistically significant.
Results
As shown in Table 1, 16 patients (11.59%) were positive, whereas the remaining 122 patients (88.41%) were negative. The number of positive patients (eight; 5.8%) was equal in both genders, with neither a statistical significant difference (p=0.212) nor correlation between gender and anti-rK39 antibodies (p=0.623), as shown in Table 1.

As shown in Table 2, the majority of positive patients belong to the age group of 23–28, 29–34, and 17–22 years. Among males, a higher number of positive patients were present in the age groups of 11–16 and 23–28 years. Among females, a higher number of positive patients were present in the age group of 35–40 years, followed by 29–34 and 23–28 years. Neither a significant difference (p=0.196) nor correlation (p=0.227) was observed between males and anti-rK39 antibodies. Moreover, neither a significant difference (p=0.514) nor correlation (p=0.302) was observed between females and anti-rK39 antibodies. There was neither a significant difference (p=0.551) nor correlation (p=0.992) between anti-rK39 antibodies, in both genders according to age groups as shown in Table 2.

As shown in Table 3, both males and females with positive anti-rK39 antibodies had normal serum TSH levels. There was no significant difference or correlation between males and TSH levels (p=0.466; 0.475), but between males and females (p=0.306; 0.275). As shown in Table 5, both males and females with positive anti-rK39 antibodies had normal serum T4 levels. Neither a significant difference nor correlation was reported between males and serum T3 levels (p=0.466; 0.475); females serum T3 levels (p=0.277; 0.283) with positive anti-rK39 antibodies, and between males and females according to serum T3 levels (p=0.306; 0.275). As shown in Table 5, both males and females with positive anti-rK39 antibodies had normal T4 levels. Only one positive anti-rK39 patient had an elevated T4 level (>12 µg/dL).

Discussion
In this study, the mean age of patients who presented with clinical signs of VL was 27.65±1.60 years. The minimum age was 5 years and maximum age was 70 years, which was consistent with results of other studies in Brazil, but were less than those of India (mean age, 45 years) [6, 14, 15]. Sixty-one patients (44.2%) were males, and the mean age was 29.65±11.10 years, which come in line with others [15]. The rest of the patients were females with a mean age of 26.12±11.89 years, minimum age of 5 years, and maximum age of 70 years, which was contrary to results of an Indian study indicated that 93% of affected patients were males. In Brazil, 65% of Kala-azar patients were males and in Ethiopia they represent (74%-85.6%) [6, 14-16]. High male patient load could be because of the economic activities that entail sex bias. Previous studies documented that males are disproportionately affected by VL compared with females; this is mainly related to their occupation (agricultural activities, owning animals, daily laborers, and soldiers) [16].

In the current study, the total number of positive patients was 115.9%, which was considered low compared with that from a study in India (44%) and only 2% in Basra southern Iraq using immunochromatography dip sticks with rK39

### Table 1. Anti-rK39 antibody test results with respect to Gender in patients with kala-azar

| Sex     | Positive No. (%) | Negative No. (%) | \(\chi^2\) | Pearson’s correlation |
|---------|------------------|------------------|------------|----------------------|
| Male    | 8 (5.8%)         | 53 (38.41%)      | 0.247      | 0.62                 |
| Female  | 8 (5.8%)         | 69 (50%)         | 0.04       | 0.623                |
| Total   | 16 (11.59%)      | 122 (88.41%)     |            |                      |

### Table 2. Anti-rK39 antibody test results with respect to the age groups of patients with kala-azar

| Age group (years) | Total No. (%) | Total Positive | Anti-rK39 antibodies | \(\chi^2\) | Pearson’s correlation |
|-------------------|---------------|----------------|----------------------|------------|----------------------|
|                   | Male          | Female         |                     |            |                      |
|                   | Positive      | Positive       |                     |            |                      |
|                   | No. (%)       | No. (%)        |                     |            |                      |
| 5-10              | (8.70%)       | (0.72%)        | (2.90%)              | 0.158      | -0.119               |
| 11-16             | (9.42%)       | (2.90%)        | (3.62%)              |            |                      |
| 17-22             | (16.67%)      | (0%)           | (4.35%)              |            |                      |
| 23-28             | (20.29%)      | (0%)           | (4.35%)              |            |                      |
| 29-34             | (19.57%)      | (1.45%)        | (11.59%)             |            |                      |
| 35-40             | (13.04%)      | (1.45%)        | (4.35%)              |            |                      |
| 41-46             | (7.97%)       | (1.45%)        | (4.35%)              |            |                      |
| 47-52             | (2.90%)       | (0.72%)        | (1.45%)              |            |                      |
| 53-58             | (0.72%)       | (0%)           | (0.72%)              |            |                      |
| 59-64             | (0%)          | (0%)           | (0%)                 |            |                      |
| 65-70             | (0.72%)       | (0%)           | (0.72%)              |            |                      |
| Total             | 138 (11.59%)  | 61 (44.20%)    | 38 (28.41%)          |            |                      |

| \(\chi^2\) | p   | Pearson’s correlation value | p   |
|------------|-----|---------------------------|-----|
| 38.571     | 0.196 | 0.51                     | 0.514 |
| 0.518      | 0.019 | 0.019                    | 0.992 |
| 0.302      | 0.628 | 0.628                    | 0.628 |
cells are suppressed during the active phase of leishmaniasis [16, 19]. The non-specific antibodies, which are a common consequence of cutaneous cellular response of lymphocytes and plasma cells, does not appear to be an effective local cellular response. In Indian patients with kala-azar, there is deranged cell-mediated immunity with a normal or exaggerated humoral immunity with a normal or exaggerated humoral response. In Sao Paulo, revealed that a normal free T4 plasma level indicates a sufficient thyroid hormone secretion for the basic needs of an organism in kala-azar patients, rejecting the possibility of thyroid hormone deficiency [20]. This supports our knowledge that continuous synthesis, storage, and release of T4 is dependent on the pulsatile stimulation of the thyroid by TSH (thyrotropin) and is a result of normal TSH levels in all patients included the current study.

Alterations in the thyroid hormone concentrations because of non-thyroid illnesses are well known in the human population. In the current study, both males and females with positive anti-rK39 antibodies have normal serum TSH and T3 levels. Neither significant differences nor correlation was present between males and females with positive anti-rK39 antibodies, and between males and females (p>0.05) with respect to serum TSH and T3 levels. Neither significant differences nor correlation was present between males (p>0.05), females (p>0.05) with positive anti-rK39 antibodies, and between males and females (p>0.05) with respect to the serum TSH and T3 levels, which were in accordance with the report from Brazil that revealed normal TSH levels among patients positive for Kala-azar [20]. In the current study, both males and females with positive anti-rK39 antibodies had normal serum T4 levels. A single patient positive for anti-rK39 antibodies had elevated serum T4 level (>12 µg/dL). Neither a significant difference nor correlation was reported between males (p>0.05), females (p>0.05) with positive anti-rK39 antibodies, and between males and females (p>0.05) with respect to serum T4 levels. These results are consistent with those of a study conducted in Sao Paulo, revealed that a normal free T4 plasma level indicates a sufficient thyroid hormone secretion for the basic needs of an organism in kala-azar patients, rejecting the possibility of thyroid hormone deficiency [20]. This supports our knowledge that continuous synthesis, storage, and release of T4 is dependent on the pulsatile stimulation of the thyroid by TSH (thyrotropin) and is a result of normal TSH levels in all patients included the current study.

### Table 3. Serum TSH levels with respect to anti-rK39 antibodies in both Genders of patients

| Parameter | Male | Female | Value | p | Value | p |
|-----------|------|--------|-------|---|-------|---|
| Normal 0.5-5.0 µU/mL | 8 (5.80%) | 50 (36.23%) | 66 (47.83%) | 0.163 | 0.686 | -0.0517 | 0.692 |
| Elevated >5.0 µU/mL | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Decreased <0.5 µU/mL | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Not detected | 0 (0%) | 3 (2.17%) | 0 (0%) | 3 (2.17%) |
| Total No. (%) | 61 (44.20%) | 77 (55.80%) |

| Parameter | Male | Female | Value | p | Value | p |
|-----------|------|--------|-------|---|-------|---|
| Normal serum (T3) 70–195 ng/dL (1.1–3.0 nmol/L) | 8 (5.80%) | 47 (34.06%) | 60 (43.48%) | 2.371 | 0.306 | 0.1407 | 0.275 |
| Elevated >195 ng/dL (>3.0 nmol/L) | 0 (0%) | 3 (2.17%) | 0 (0%) | 9 (6.52%) |
| Decreased <70 ng/dL (<1.1 nmol/L) | 0 (0%) | 3 (2.17%) | 0 (0%) | 0 (0%) |
| Total | 61 (44.20%) | 77 (55.80%) |

### Table 4. Serum T3 level with respect to anti-rK39 antibodies in both Genders of patients

| Parameter | Male | Female | Value | p | Value | p |
|-----------|------|--------|-------|---|-------|---|
| Normal 0.5-5.0 µU/mL | 8 (5.80%) | 50 (36.23%) | 66 (47.83%) | 0.163 | 0.686 | -0.0517 | 0.692 |
| Elevated >5.0 µU/mL | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Decreased <0.5 µU/mL | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Not detected | 0 (0%) | 3 (2.17%) | 0 (0%) | 3 (2.17%) |
| Total No. (%) | 61 (44.20%) | 77 (55.80%) |

In kala-azar, there is deranged cell-mediated immunity with a normal or exaggerated humoral response. In Indian patients with kala-azar, there does not appear to be an effective local cellular response of lymphocytes and plasma cells, which are a common consequence of cutaneous allergic form of leishmaniasis [16, 19]. T cells are suppressed during the active phase of the disease [16, 19]. The initial response to kala-azar appears to stimulate both specific and non-specific increases of immunoglobulins (hyper-gammaglobulinemia). The specific response to leishmania antigens is not protective. The non-specific increase of immunoglobulins in patients with kala-azar may be the result of deviation to the systemic lymphoreticular system of antigens, which are normally mopped up by the Kupffer cells of the liver. The non-specific antibodies may remain elevated up to 1 year even after complete cure; hence, serological tests may not be able to discriminate between current and past infections among newly cured patients [19]. The majority of positive patients belongs to the age group 23–28, 29–34, and 17–22 years. Among males, a higher number of positive patients were present in the age groups of 11–16 and 23–28 years. Among females, a higher number of positive patients were present in the age groups of 35–40 years, followed by 29–34 and 23–28 years. This is in accordance with the results of an Ethiopian study in which a higher number of patient with VL were recorded in the age groups of >14 years old. Also come in contrast to another study in South Sudan, in which 56% of patients were at the age group of <5 years [16, 18]. In the current study, there was neither significant differences (p=0.196) nor correlation (p=0.227) between male age group distribution and anti-rK39 antibodies (p=0.227). Furthermore, there was no significant difference (p=0.514) or correlation between female age group distribution and anti-rK39 antibodies (p=0.302). Neither significant differences (p=0.551) nor correlation (p=0.992) was reported between sexes with respect to the age group distribution and anti-rK39 antibodies, which are similar to results of other studies [8, 16, 17].
The serum levels of T4 remain at a normal range in majority of positive patients for anti-rK39 antibodies. Increased T4 levels in serum reflect the possibility that L. donovani initiates the development of a state of hyperthyroidism even in a limited number of infected patients (0.72%), mainly among males by induction of neurological stimuli for the hypothalamus–pituitary–thyroid axis leading to accelerating of the thyroglobulin proteolysis, which causes the release of thyroxine (T4) and triiodothyronine (T3) into the blood within 30 min \[^{21, 22}\].

One of important limitation of this study was the relatively small sample size because of the absence of public funding, and this reflects that the less number of seropositive patients as a result of public funding, and this reflects that the less number of patients were investigated according to the budget. To overcome this obstacle, we recommended a large-scale study of cured cases of visceral leishmaniasis in north-western Ethiopia. Parasites & vectors 2014; 7: 470.

This study concludes that Anti-rK39 antibodies, a diagnostic marker for visceral leishmaniasis have no correlation with patients age and gender. Serum TSH and T3 levels were not affected by VL, but VL causes an increase in serum T4 levels. Thyroid involvement appears to be uncommon in patients with VL.

### Table 5. Serum T4 level with respect to anti-rK39 antibodies in both Genders of patients

| Parameter                          | Male | Female |
|------------------------------------|------|--------|
|                                    | Anti-rK39 Antibodies | Anti-rK39 Antibodies | Anti-rK39 Antibodies | Anti-rK39 Antibodies |
| Positive                           | 7 (5.07%) | 8 (5.80%) | 50 (36.23%) | 58 (40.03%) |
| Elevated >12 µg/dL (>155 nmol/L)   | 1 (0.72%) | 0 (0%) | 3 (2.17%) | 11 (7.97%) |
| Decreased <5 µg/dL (<64 nmol/L)    | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Total                              | 61 (44.20%) | 77 (55.80%) |                |                |
| Pearson’s correlation               | -0.0932 | 0.139 | 0.475 | 0.228 |

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