

Hidden Antibody Testing: Assessing Immune Response Against Surface Antigens of the Parasite *Leishmania Donovanii* using ELISA

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ABSTRACT

Background: Visceral leishmaniasis remains important tropical disease of high morbidity and mortality, especially in regions like Southern Iraq. Serological tests like ELISA for antigens like rK39 were encouraging but require validation at the regional level and correlation with disease severity investigated. **Aim of the Study:** To evaluate immune reactivity to *L. donovani* surface antigen rK39 using ELISA in patients suspected of having VL. And to establish correlations of anti-rK39 antibodies levels, organ enlargement (splenomegaly, hepatomegaly), and disease severity to enhance diagnosis and prognosis. **Methods:** The case-control study included 90 cases of Visceral leishmaniasis confirmed by parasitological examination and 90 controls matched for region, sex, and age. Venous blood samples were drawn, and serum anti-rK39 antibodies were measured using a standardized recombinant antigen ELISA protocol. Clinical assessment of spleen and liver volumes was performed by clinical examination and ultrasound. **Results:** VL patients showed higher concentrations of anti-rK39 antibodies (150 ± 50 ng/ml) compared to the control group (12 ± 5 ng/ml, $P < 0.001$). A strong positive correlation was found between antibody titers and spleen size ($P < 0.001$), indicating that patients with larger spleens had more antibodies. A moderate correlation was also found with liver size ($P = 0.025$). From these results, it is clear that antibody levels are an indicator of disease severity. **Conclusion:** The ELISA test for detecting anti-rK39 antibodies is a highly sensitive and specific diagnostic method for active visceral leishmaniasis. Antibodies are also associated with organ enlargement, suggesting their usefulness in measuring disease severity and course.

Keywords: *Leishmania Donovanii*, Visceral Leishmaniasis, Anti-Rk39, Immune Response

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar, the "red plague," is an acute systemic parasitic disease most commonly caused by *Leishmania* *Donovan* and *Leishmania* *infantum*. Transmission is by infected female *Phlebotomus* sand fly bites and results in one of the most incapacitating of the neglected tropical diseases. VL epidemicity is most prevalent in tropical and subtropical regions, with global distribution widespread in East Africa, South Asia, the Mediterranean, and Latin America. In Iraq and other Arab countries, VL remains an important public health threat, particularly in rural, socioeconomically underprivileged populations, where healthcare is not well covered, and no effective vectors exist [1, 2]. Clinically, VL manifests as continuous fever, splenomegaly, hepatomegaly, weight loss, and pancytopenia. If left untreated, infection is invariably fatal, as it results from secondary infection as well as extensive immunosuppression [3]. The high mortality and morbidity of VL indicate the extreme need for earlier and accurate diagnosis methods to improve patients' prognoses as well as preventing transmission. The conventional methods such as microscopic examination, culture, or symptomatology of bone marrow or spleen aspirate are invasive, time-consuming, or not highly sensitive, especially in co-infected or asymptomatic patients [4, 5]. Such limitations have spurred the shift to modern serological and immunological diagnostic methods, predominantly enzyme-linked

immunosorbent assay (ELISA) and rapid diagnostic tests (RDT) that identify host antibodies to parasite antigens. Of antigens employed for serodiagnosis, recombinant K39 (rK39) antigen—a kinesin-related protein from *L. chagasi*—is one that is generally accepted due to high sensitivity and specificity in most endemic regions [3, 6]. The rK39-ELISA and immunochromatographic tests have facilitated simplification of VL diagnosis, especially under field circumstances. Diagnostic sensitivity is, however, geographical in nature due to antigenic heterogeneity of *Leishmania* species and host immune heterogeneity [1, 7]. Remarkably, rK39-negative VL has been documented in endemic regions, calling for region-specific validation and potential search for novel or supplementary antigens [8, 9]. Other *L. Donovan* antigens such as KDDR-plus, LDBPK_323600.1, Aurora kinase, and KR95 have also been proposed as potential candidates for improved serodiagnosis [10-12]. Apart from this, inclusion in an ELISA platform has also revealed improved diagnostic resolution in human and canine VL, as well as immunocompromised or co-infected patients [13, 14]. In this regard, there is a pressing need to assess rK39-ELISA's diagnostic utility within Iraq and other similarly situated local contexts. Though rK39 has been calibrated outside of these locations, sensitivity and specificity to local parasite isolates and to local presentations of disease need to be evaluated urgently. Correlation of

reactivity in the assay to known markers of disease such as level of organomegaly could add further interpretability to the assay, as well as provide insight into immune status and disease outcomes.

Aim of the Study

The study aims to evaluate *L. Donovanii* rK39 antigen-induced humoral immunity by means of ELISA in VL suspects. Specifically, it aims to (1) establish sensitivity and specificity of ELISA in our setting, and (2) investigate correlation of levels of antibodies and the degree of splenomegaly and hepatomegaly. Findings can be used to enhance diagnostic protocols and aid in case management in endemic regions.

MATERIALS AND METHODS

Study Design and Participants

A case-control study of 180 participants from Southern Iraq, 90 confirmed VL patients, and 90 healthy, age- and sex-matched control participants were conducted. The study was conducted from 1st August 2024 to 10th May 2025. Molecular diagnosis of VL was done by PCR. Controls were selected from the same study area who did not exhibit any symptom or sign of infection or any infectious or autoimmune, chronic, or immunosuppressant disease. Institutional review board ethics approval and informed consent were taken from all the participants.

Sample Collection

Five mL of blood was drawn by sterile venipuncture, and serum was separated by centrifugation of 3000 rpm for 10 minutes. Aliquot preservation was done at -80°C to prevent degradation, waiting for analysis.

Recombinant rK39 Antigen Preparation

Leishmania donovani rK39 gene fragment was cloned into an expression plasmid, which was transformed to *Escherichia coli* BL21 (DE3). The protein was expressed following induction by 0.5 mM IPTG at 37°C for 4 hours. His-tagged recombinant protein was purified using nickel-affinity chromatography and purity determined by SDS-PAGE and Western blotting using His antibodies. The antigen was dialyzed in PBS, aliquotted, and stored at -20°C . Bradford assay was used to measure protein concentration.

ELISA Optimization and Validation

Pre-test routine optimization of the ELISA process included antigen coating concentration, serum dilutions, time of incubation, and utilized blocking reagents. To check assay specificity, sera of patients showing other infectious diseases, for example, tuberculosis and malaria, were screened to identify cross-reactivity. Intra-assay as well as inter-assay variability were ascertained from repeat measurements using CVs that were maintained below 10% and 15%

respectively. Standard curves were constructed using two-fold serial dilutions of pooled positive control sera as calibrated to that of a reference standard.

ELISA Procedure

- Plate coating: Greiner Bio-One MICROLON® 600 high-binding 96-well plates were covered overnight at 4°C using 100 μL /well of rK39 antigen 1–5 $\mu\text{g}/\text{mL}$ in carbonate-bicarbonate buffer (9.6 pH)
- Washing: Plates were washed thrice in PBS-T (PBS + 0.05% Tween-20) and were blocked using 200 μL of 5% BSA in PBS-T for 1 hour at 37°C
- Sample Incubation: Diluted serum samples were diluted to 100-fold in PBS-T. 100 μL of diluted samples were added into duplicate wells and incubated at 37°C for 1 hour.
- Washing: PBS-T was used to wash wells five times
- Secondary Antibody: Secondary antibody of anti-human IgG, diluted to 1:10,000, and incubated at 37°C
- Substrate development: Washing was succeeded by addition of 100 μL of TMB substrate and development in the dark for 10–20 min. The reaction was interrupted by 50 μL of 0.16 M H_2SO_4 .
- Measuring: OD was measured at 450 nm using a microplate reader. Controls and samples were performed in duplicate, and mean OD was computed for analysis.

Determining Diagnostic Performance and Cut-point

The cut-point was established as negative control mean of OD + two standard deviations. Receiver Operating Characteristic (ROC) curve analysis was performed to establish the optimal cut-point value, sensitivity, specificity, and also AUC (area under ROC curve) using 95% confidence intervals.

Organ Size Measurement

Standard percussion and palpation were used to measure spleen and liver sizes clinically in centimeters, which were confirmed by ultrasonography wherever possible. The sizes of organs were also classified into preagreed ranges for correlation studies.

Statistical Analysis

Shapiro-Wilk test was used to establish normality of data. Student's t-test or Mann-Whitney U test was used to compare quantitative variables (antibody titers). Pearson or Spearman correlation coefficients were used to establish correlations of antibody titers and organ sizes. One-way ANOVA or Kruskal-Wallis tests were used to compare more than two groups, and post hoc tests were used as needed. P-values < 0.05 were taken to be statistically significant. All analysis was executed using SPSS (26).

Results

Table 1: (A) Demographic Composition of Study Group

Variable	Visceral Leishmaniasis Patients (N = 90)	Control Group (N = 90)	P-Value
Gender			
Males	50	48	0.835
Females	40	42	
Age Group			
Under 18 years	25	30	0.576
18 to 40 years	30	28	
Over 40 years	35	32	

Table 1: (b) Demographic Composition of Study Group

Variable	Visceral Leishmaniasis Patients (N = 90)	Control Group (N = 90)	P-Value
Geographical Location			
Southern Iraq	90	90	-

Demographic analysis showed that groups of visceral leishmaniasis (VL) patients and controls were well matched both for sex and distribution of ages. Among 90 VL patients, 55.6% were male and 44.4% were female, compared to 53.3% male and 46.7% female of the control group, which was not significant, as indicated by p-value of 0.835.

Similarly, age was also classified into three categories: below 18 years, 18 to 40 years, and above 40 years of ages. Patient group included 27.8%, 33.3%, and 38.9% of each of these respective groups,

closely resembling that of control (33.3%, 31.1%, and 35.6% respectively) and an otherwise non-significant p-value of 0.576.

All participants were from Southern Iraq, and hence it ensured homogeneity of exposure environment as well as of the ecology of vectors. All these results establish these groups to be demographically similar to each other, avoiding any confounding due to geography, sex, or age and creating an appropriate platform to compare immunological parameters of infected individuals to uninfected controls.

Table 2: Anti-rK39 Antibody Titers in VL Subjects and Controls

Variable	Visceral Leishmaniasis Patients (N = 90)	Control Group (N = 90)	P-Value
Antibody Level Against rK39 (ng/mL)			
Mean (ng/mL)	150 ± 50 ng/mL	12 ± 5 ng/mL	< 0.001
Minimum (ng/mL)	75 ng/mL	5 ng/mL	
Maximum (ng/mL)	350 ng/mL	25 ng/mL	
Antibody Distribution (Percentile)			
0 - 50 ng/mL	5 (5.56%)	85 (94.44%)	< 0.001
51 - 100 ng/mL	15 (16.67%)	5 (5.56%)	
101 - 150 ng/mL	45 (50%)	0 (0%)	
> 150 ng/mL	25 (27.78%)	0 (0%)	

Quantitative measurement of titre of antibodies to *Leishmania donovani* rK39 antigen in serum proved to be of extreme value in differentiating VL patients from normal control persons. The titre of patients' antibodies was mean = 150 ± 50 ng/mL, which was very much higher than that of 12 ± 5 ng/mL of normal control persons, $p < 0.001$. The range of titre of antibodies in patients was 75 to 350 ng/mL, whereas in control persons it was 5 to 25 ng/mL, which shows an obvious immunological discrimination. If patient groups were classified by titre of antibodies, it was observed that 94.44% of control

persons were of titre values below 50 ng/mL, whereas merely 5.56% of patients were below it.

On the other hand, half of patients (50%) were of moderate titre (101–150 ng/mL) and nearly 28% were of titre values above 150 ng/mL. None of control persons were of titre values above 100 ng/mL. This result strongly confirms rK39 ELISA sensitivity and specificity for active VL infection, and also establishes an apparent role of titre of antibodies as a marker for disease status or antigen burden of that disease.

Table 3: Relationship of Spleen Size to Antibody Level

Variable	< 10 cm (N = 10)	10-15 cm (N = 30)	> 15 cm (N = 50)	P-Value
Spleen Size (cm)	-	-	-	-
Antibody Level Against rK39 (ng/mL)	75 ± 20 ng/mL	115 ± 28 ng/mL	145 ± 35 ng/mL	< 0.001

Correlation of anti-rK39 antibody titers and spleen sizes in VL patients was statistically significant, and it was proportional to levels of spleen enlargement. The decreased mean titers of antibodies, 75 ± 20 ng/mL, were observed in patients whose spleen measurements were below 10 cm (N = 10). The moderately splenomegalic (10–15 cm, N = 30) patients were observed to have an increased mean titer of 115 ± 28 ng/mL. The maximum mean titer of antibodies, 145 ± 35 ng/mL, was observed in the case of massive splenomegaly of more than 15 cm

(N = 50). p-value of < 0.001 indicates that there is high positive correlation. The observation suggests that as parasite load and consequent immunopathological manifestations in spleen increase, it is reflected in increase of corresponding humoral immunity, i.e., antibody response to parasite surface antigens, e.g., rK39 as well. The observation is in accordance with well-established immunobiology of VL, where spleen stands at center of antigen presentation as well as of antibody synthesis in case of chronic infection.

Table 4: Antibody Level and Liver Size Relationship

Variable	< 12 cm (N = 10)	12-18 cm (N = 35)	> 18 cm (N = 45)	P-Value
Liver Size (cm)	-	-	-	0.025
Antibody Level Against rK39 (ng/mL)	70 ± 22 ng/mL	125 ± 30 ng/mL	145 ± 38 ng/mL	

The same, though weaker, correlation was also discovered to be present between liver enlargement and anti-rK39 levels of antibodies in VL patients. In patients whose liver measurement was below 12 cm (N = 10), the mean antibody level was 70 ± 22 ng/mL. This was higher in patients with moderate enlargement of the liver (12–18 cm; N = 35) to a level of 125 ± 30 ng/mL. The mean antigenic concentration

of antibodies (145 ± 38 ng/mL) was maximum in patients whose liver sizes were more than 18 cm (N = 45). The p-value of 0.025 is significant, which shows that there is correlation. The correlation is suggestive of the fact that liver enlargement in VL, more commonly due to lymphoid hyperplasia, and activation of Kupffer cells, is also linked to elevated levels of antibodies in the body. Though not as

strong, as that of splenic enlargement, this correlation still goes to illustrate association of organ involvement and degree of host's humoral immunity.

Discussion

The study exhibited much higher titers of anti-rK39 antibodies among VL patients than in an equivalent demographic control group. The mean titer of antibodies among VL patients was 150 ± 50 ng/mL, much higher than 12 ± 5 ng/mL among controls ($p < 0.001$). The results confirm previous studies re-establishing rK39-based serological tests as an excellent gold standard for VL diagnosis, particularly among endemic populations [3, 6]. The diagnostic utility of rK39 antigen has been established beyond any doubt. Hagos *et al.* [6] conducted meta-analysis in Ethiopia and proved that, among symptomatic patients, rK39 RDT is both sensitive as well as specific, as clear from our finding that no control individuals crossed 50 ng/mL when titers of antibodies are taken into consideration. Similarly, Getnet *et al.* [3] proved that rK39 RDT is of excellent diagnostic accuracy, as clear from our finding that 94.44% of normal controls were below 50 ng/mL of antibodies and 77.78% of VL patients were above 100 ng/mL of antibodies. Limitations to universality of use of rK39-based tests were, however, observed. Deepachandi *et al.* [7] defined rK39-seronegative VL among Sri Lankan endemic foci and possible strain variation of *Leishmania donovani* and need to utilize supplementary or alternate antigens. This would be of lesser significance to us in Southern Iraq, where high titers among VL patients suggest strong and uniform serological reactivity to rK39.

The close correlation of rK39 antibody titers to liver and spleen sizes also illustrate the utility of this marker in clinics. Antibody titers also correlate with organomegaly, as mean antibody titers of 145 ± 35 ng/mL of spleen sizes >15 cm were higher than 75 ± 20 ng/mL of spleen sizes <10 cm ($p < 0.001$). The same trends were also true for liver sizes as well. The findings also illustrate that there is an association of disease burden to antibodies, as described by Roberts *et al.* [11] and Ramos *et al.* [13] that defined that intensity of serology is generally associated with parasite load and disease severity. While rK39 remains a useful ongoing target, further studies extended and discovered novel diagnostic antigens. Iniguez and Kamhawi [9] and Siqueira *et al.* [10] described antigens like KDDR-plus and other kinesin-derived antigens that were more sensitive, especially among co-infected or early-infection patients. *Leishmania donovani* Aurora kinase and repressor of differentiation kinase 2 were also discovered by Bhattacharyya *et al.* [8] as novel markers complementing rK39-based

tests and yielding diagnostic breadth, especially in rK39-negative or atypical presentations. In addition, it was also evidenced by field studies that performances of the tests can vary based on endemicity and host determinants. Eyayu *et al.* [15] showed appropriate application of rK39 in urine samples, which, though, was cautioned against by Nail *et al.* [16] by warning that hematologic malignancies reduce the specificity of rK39 and direct agglutination tests, leading to false positives. Although in our population these confounding elements were not present, these findings need to be remembered while creating future algorithms, particularly in populations where comorbidities overlap.

Conclusions

1. The study conclusively shows that VL patients possess elevated levels of anti-rK39 antibodies when compared to normal controls, justifying that diagnosis based on ELISA is specific and an effective diagnostic method in active visceral leishmaniasis.

2. The strong correlation of spleen size to antibody titers suggests that disease severity and parasite load correlate to levels of humoral immunity, where increased spleen sizes relate to higher levels of antibodies.

3. The strong correlation of liver enlargement with raised antibodies indicates liver involvement in VL as a component of general activation of immunity, albeit to a comparatively lesser degree than splenomegaly.

4. Demographic features of age, sex, and geography were also matched across groups to ensure that any immunological variation observed was due to disease status rather than confounding covariates.

Ethical Statement

The study was conducted following approval from the College of Health and Medical Technology/Kufa, Al-Furat Al-Awsat Technical University Institutional Review Board (IRB). Informed written consent was provided by all of the participants prior to enrollment based on the Declaration of Helsinki.

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