Cross-Reaction of Sera from Patients with Various Infectious Diseases with *Leishmania infantum*

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Key Words

Visceral leishmaniasis · Malaria · False-positive reactions · Indirect fluorescent antibody · Counterimmunoelectrophoresis

Abstract

Objective: To compare and evaluate the application of indirect fluorescent antibody (IFA) and counterimmunoelectrophoresis (CIEP) for laboratory identification of visceral leishmaniasis. **Materials and Methods:** Serum samples from patients with malaria (*Plasmodium vivax*, n = 86; *Plasmodium falciparum*, n = 38), brucellosis (n = 26), tuberculosis (n = 31) and typhoid fever (n = 35) were examined for the presence of antibody to *Leishmania infantum* antigen using IFA and CIEP tests. **Results:** Using IFA, false-positive results were malaria (*P. vivax* 19.8%, *P. falciparum* 13.2%), tuberculosis (6.4%), brucellosis (3.8%), and typhoid fever (2.8%). Using CIEP, a lower percentage of false-positives was observed only among malaria patients (*P. vivax* 2.3%, *P. falciparum* 2.6%). Serum samples from patients with other infectious diseases were negative in the CIEP test. **Conclusion:** Based on the results of this study, the CIEP technique is recommended for immunodiagnosis of visceral leishmaniasis, especially in regions where malaria, brucellosis and tuberculosis are prevalent.

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Introduction

Visceral leishmaniasis (VL) is an endemic disease in various parts of Iran [1–3]. This disease is caused by *Leishmania infantum*, which mostly affects the pediatric age group and is usually characterized by prolonged fever, hepatosplenomegaly, reversed albumin-to-globulin ratio and proteinuria [4]. Currently, the definite diagnosis of VL is based on the microscopic demonstration or isolation of the parasites from bone marrow or spleen biopsy. This procedure, although specific, lacks enough sensitivity, and hence specimens with low density parasites may be overlooked. Moreover, the technique is invasive and hazardous to the patients and therefore is not recommended [5, 6]. Various immunodiagnostic procedures including counterimmunoelectrophoresis (CIEP), direct agglutination test on the serum and urine, enzyme linked immunosorbent assay and indirect fluorescent antibody (IFA) with various degrees of sensitivity and specificity are widely used for immunodiagnosis of this disease [7–12]. Recently a rapid K39 strip test has been developed and found to have high sensitivity and specificity in the diagnosis of Indian VL [13]. Polymerase chain reaction, though highly sensitive, is costly and facilities for performing the test are not always available [14–16]. Since IFA and CIEP techniques are widely used for immunodiagnosis of VL in this region, we sought to evaluate these

procedures using serum samples from patients with various infectious diseases as the source of antibody and *L. infantum*, the etiological agent of VL, as the antigen.

Materials and Methods

Patients

A total of 263 serum samples from patients with various infectious diseases including malaria (n = 124; *Plasmodium vivax*, n = 86; *Plasmodium falciparum*, n = 38), typhoid fever (n = 35), pulmonary tuberculosis (n = 31), syphilis (n = 25), brucellosis (n = 26), VL (n = 22), and 50 samples from normal healthy individuals as negative control were used in this study.

Patients with malaria, VL, tuberculosis and typhoid fever were diagnosed by demonstration of their etiological agents combined with clinical signs and symptoms. The 26 patients with brucellosis, 12 acute and 14 chronic, were identified by clinical signs and symptoms combined with either isolation of *Brucella melitensis* (11 cases at the acute stage) from blood culture, or by standard tube agglutination test. All patients in this group revealed a significant diagnostic serum titer of $\geq 1:160$. The syphilitic patients were diagnosed on the basis of history, clinical features and positive standard VDRL test on the undiluted sera. Normal serum of the negative control group and samples from patients with various infectious diseases (except VL) who had a history of leishmaniasis were not included in this study.

Leishmania Antigen

L. infantum was obtained from Dr. G.H. Edrissian, School of Public Health, Tehran, Iran. The organism has been isolated from a patient with VL and maintained on NNN media. The parasite was subcultured on Panmed medium in the presence of 10% fetal calf serum. The promastigotes were harvested in their logarithmic growth phase and washed in phosphate-buffered saline (PBS, pH = 7.2). Particulate and soluble antigens for IFA and CIEP tests were then prepared as described elsewhere [4, 17].

Counterimmunoelectrophoresis

CIEP was carried out on all heat-inactivated, undiluted serum samples of patients and normal individuals using the soluble form of *L. infantum* as antigen. This technique was carried out on a 13 × 8 cm glass plate covered by 1% molten agarose (Hoechst, Germany). Rows of 2 wells were punched 5 mm apart and the serum samples were placed in the anodal and soluble antigen in the cathodal wells. The plate was then subjected to electrophoresis (Austigen Apparatus, Hyland, Calif., USA) using acetate buffer, pH = 8.2 at 40 mA for 30 min and examined for the presence of precipitin band/s. Development of 1–2 precipitin bands between the two wells indicate positive CIEP test. In both CIEP and IFA tests serum samples from normal healthy individuals and parasitologically proven cases of VL were used as negative and positive controls, respectively.

Indirect Fluorescent Antibody

The test was performed according to Kohanteb et al. [17] and Shiddo et al. [18]. Briefly, the spot smear of acetone-fixed promastigotes of *L. infantum* were coated with various dilutions of serum and incubated at 37°C for 30 min followed by 1-min washing in

Table 1. Comparison of the percentage of false-positive reactions encountered in IFA with CIEP test using *L. infantum* as antigen

Diseases	Tested sera	Number (%) positive in	
		IgG IFA	CIEP
Malaria			
<i>P. vivax</i>	86	17 (19.8)	2 (2.3)
<i>P. falciparum</i>	38	5 (13.1)	1 (2.6)
Brucellosis	26	1 (3.8)	0 (0)
Tuberculosis	31	2 (6.4)	0 (0)
Typhoid fever	35	1 (2.8)	0 (0)
Syphilis	25	0 (0)	0 (0)
VL	22	22 (100)	22 (100)
Normal serum	50	0 (0)	0 (0)

PBS (pH = 7.2). The slides were overlaid by diluted antihuman IgG globulin conjugated with fluorescein isothiocyanate for 30 min and washed in PBS (pH = 7.2). The slides were examined under a fluorescent microscope and the fluorescence intensity of *Leishmania* promastigotes was estimated and graded as 1+ to 4+ at the corresponding serum dilutions.

Results

The comparisons of false-positive results using IFA and CIEP tests are shown in table 1. A higher percentage of false-positive reactions were encountered in the serum samples from patients with malaria, brucellosis, tuberculosis and typhoid fever in IFA than in CIEP test. Sera of syphilitic patients and normal individuals were negative in CIEP test but showed antibody titer ranging from 1:8 to 1:64 in the IFA test. The IFA titer of 22 parasitologically proven cases of VL varied from 1:128 to 1:1,024. The results of IFA tests carried out on serum dilutions of patients with various infectious diseases using promastigotes of *L. infantum* as antigen are shown in table 2. The antibody titer of $\geq 1:128$ was of diagnostic value for VL. On this basis, 19.8% of patients infected with *P. vivax* and 13.2% with *P. falciparum* showed false-positive reactions in IFA tests. Lower percentage of positive results were also observed among patients with tuberculosis (6.4%), typhoid fever (2.8%) and brucellosis (3.8%).

Discussion

Indirect immunofluorescent antibody and CIEP are the two most valuable techniques for the laboratory diagnosis of VL in Iran [19]. Significant cross-reactivity of

Table 2. Distribution of patients and control serum results in the *L. infantum* IFA test

Diseases	Tested sera	Percent of sera showing positive IgG-IFA end point titers of					
		1:16	1:32	1:64	1:128 ^a	1:256	≥ 1:512
Malaria							
<i>P. vivax</i>	86	34.9	31.4	13.9	12.8	5.8	1.2
<i>P. falciparum</i>	38	42.1	34.2	10.5	5.3	5.3	2.6
Brucellosis	26	57.7	23	15.4	3.8	0	0
Tuberculosis	31	54.8	32.2	6.4	3.2	3.2	0
Typhoid fever	35	54.3	28.6	14.3	2.8	0	0
Syphilis	25	60	32	8	0	0	0
VL	22	0	0	0	27.3	63.6	9.1
Normal serum	50	56	44	0	0	0	0

^a Serum titer ≥1:128 is considered positive.

malaria sera with *Leishmania donovani* antigen in IFA technique as observed in this study has also been reported by other investigators [20, 21]. Abramo et al. [22], using the same technique, have shown that the sera of 62% of patients with visceral and 38% with cutaneous leishmaniasis cross-reacted with *P. falciparum* blood stage antigen. In the present study, a lower percentage of cross-reactivity was noted among patients with tuberculosis (6.4%), brucellosis (3.8%) and typhoid fever (2.8%). The IFA test could clearly detect and differentiate the 22 parasitologically proven cases of VL as the antibody titer among these patients was not less than 1:128 and moreover the titer did not exceed 1:32 among 50 normal healthy individuals. Although the test is highly sensitive, the cross-reactivity described here reduces the specificity of the test. Less cross-reactivity was observed with CIEP than IFA since the 22 sera of VL patients were positive in 2.6 and 2.3% among patients with *P. falciparum* and *P. vivax*, respectively (table 1). Moreover, sera of patients with other diseases and of normal healthy controls were nonreactive with *L. infantum* soluble antigen in the CIEP test as reported by Aikat et al. [23] and Sinha and Sehgal [24]. These authors stated that the false positivity could be eliminated if the amastigote soluble antigen of *Leishmania* is used in the CIEP test [23, 24]. Using the K39 strip test, Goswami et al. [13] did not find any false-positive results among a limited number of serum samples from patients with malaria and tuberculosis. This test is believed to be highly sensitive (100%) and specific (98.18%) for the diagnosis of VL in India. However, the K39 strips are not always available and the test needs further evaluation in regions where malaria, tuberculosis and brucellosis are prevalent.

Leishmania and *Mycobacteria* spp. share some common antigens [25, 26], but such antigenic similarities between plasmodia and *Leishmania* are not reported, although the two genera are phylogenetically closer. Non-specific elevation of immunoglobulins in both malaria [27–29] and VL is reported [30, 31], which could be an explanation for the false-positive results among malaria patients.

Conclusion

Based on the results of this study, the CIEP technique is recommended for immunodiagnosis of VL, especially in regions where malaria, brucellosis and tuberculosis are prevalent.

Acknowledgments

The authors would like to thank the Vice Chancellor for Research, Shiraz University of Medical Sciences for financial support of this project (grant No. 78–791). The authors are also grateful to Prof. F. Hanjani for his critical comments on the manuscript. The technical assistance of Mr. M. Hosseini Farzad is greatly appreciated. The authors are also thankful to Miss S. Golbon for her secretarial assistance.

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