

Serodiagnosis of cutaneous leishmaniasis in the Syrian Arab Republic

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ABSTRACT

الهدف: تقييم فاعلية اختباري التبرص المناعي (WB) والمقايسة المناعية الأنزيمية (ELISA)، باستخدام بروتينات مستضدية محضرة من الأشكال أمامية السوط لطفيلي الليشمانية الجلدية المستنبت مخبرياً على أوساط صناعية، لتشخيص داء الليشمانية الجلدي في سورية.

الطريقة: أجريت الدراسة على 290 عينة مصلية مأخوذة من مصابين أو مشكوك بإصابتهم بالداء الجلدي، ومن أشخاص أصحاء خلال الفترة ما بين 2002م وحتى 2005م. حيث طبق الاختبارين المصلين على هذه العينات المصلية في مختبرات جامعة دمشق - دمشق - سوريا.

النتائج: لوحظت الأضداد الموجهة ضد طفيلي الليشمانية في 250 عينة (92.5%) و 254 عينة (94%)، عند تطبيق اختباري الاليزا (ELISA) والتبرص المناعي (WB) على التوالي. كما لوحظ وجود ارتباط بين شدة تركيز هذه الأضداد وعدد الآفات الجلدية الموجودة عند المصاب.

خاتمة: بينت نتائج الدراسة عدم وجود فرق معنوي بين اختباري المقايسة المناعية الأنزيمية (ELISA) والتبرص المناعي (WB). ونظراً لسهولة تطبيقها، يمكن استخدامها في تشخيص داء الليشمانية الجلدي في سورية.

Objective: To evaluate the validity of western blot (WB) and enzyme linked immunosorbent assay (ELISA) that use antigens from culture promastigote from *Leishmania* parasites, for laboratory diagnosis of cutaneous leishmaniasis in Syria.

Methods: We utilized 290 serum samples from endemic areas (patients group) and other regions (control samples) in Syria during 2002-2005 and the serological testing was brought to the applied the serological tests at the Department of Animal Biology, Damascus University, Damascus, Syria.

Results: Anti-*Leishmania* antibody was detected in 250 (92.5%) cases using the ELISA and 254 (94%)

cases using the WB. It is also noted that this response can change according to the number of lesions.

Conclusion: Results of this study showed that there was no significant difference between ELISA and WB, which are easy to perform. Thus, they can be used for diagnosing the cutaneous leishmaniasis in Syria.

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Protozoan parasites of the genus *Leishmania* cause a spectrum of diseases found all over the world in tropical and subtropical regions. The severity of disease varies begging self-healing cutaneous leishmaniasis to sever muco-cutaneous or visceral leishmaniasis.¹ Cutaneous leishmaniasis (CL) is highly endemic in the north and east Mediterranean regions in the Syrian Arab Republic, with more than 75.9% of all CL cases recorded from these regions. During the 8-year-period (2000 and 2007), the number of cases reported by the Department of Disease Control, Ministry of Health was: 19,837 in 2000; 24,839 in 2001; 21,560 in 2002; 28,881 in 2003; 26,878 in 2004; 21,984 in 2005; 18,741 in 2006 and 17,709 in 2007. Before the 1960s, CL (known as Aleppo boil) was endemic in the north region of Syria and was characterized by anthroponotic epidemic.^{2,3} After the implementation of a campaign against mosquitoes aimed at controlling malaria, the rate of CL declined considerably between 1962 and 1971.⁴ The number of CL cases reported by the Department of Disease Control during the 18-year period (from 1990-2007) was 311760 in Syria. The reported incidence reached a peak in 2003 with

28881 cases. There was a considerable reduction in the number of reported cases in 1998, but the number of reported cases increased again in 1999 and continued for the coming years. The majority (53%) of CL cases are still being reported from the north region of Syria. The definitive diagnosis of CL relies on the clinical manifestations of the disease together with the detection of the intracellular stages of the parasite by examination of skin lesion smears and culturing of specimens.⁵ The diagnosis and treatment centers were established in each province of Syria, and a standard treatment regimen with Antimonials (Glucantime) was started for patients with parasitologically proven CL. However, often the presumptive diagnosis cannot be confirmed by the identification of the parasite, in this case, sero-diagnosis appears to be a valid diagnostic alternative.⁶⁻¹⁰ During the past few years, particular emphasis has been given to characterization of *Leishmania* antigenic components with a goal of identifying specific antigens for diagnosis. In CL, anti-*Leishmania* antibodies may be detected in serum, although normally they are present at low levels.⁹⁻¹³

The purpose of this study is to determine the antibody responses using 2 serological tests, Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot (WB), of sera obtained from non-treated CL patients with different characterized clinical aspects (lesion age, size, and number) in different areas in Syrian Arab Republic, in order to support the classical diagnostic methods, and to determine the effects of clinical differences on the antibody responses.

Methods. In this study, we utilized 290 serum samples from patients from endemic areas and control samples from other regions in Syria during 3 years (2002-2005). An accepted questionnaire with epidemiological data by the Department of Disease Control in Syria (age, gender, address, lesion number, size, and age and so forth) was filled out for each patient. The patients were notified on all the procedures and signed informed consent was obtained. The Ethics Committee of the Damascus University Science Faculty, Damascus, Syria approved the study. The serum samples used were obtained from the following: 1. Non-treated CL patients parasitologically confirmed [nodular, ulcerative, or popular lesion, and demonstrated to have *Leishmania* parasites in smears from skin lesion and culture samples (n=225)]. 2. Patients clinically suspected of CL, however, no *Leishmania* parasites were observed in both smears and culture (n=45). 3. Health individuals from CL endemic regions (n=10). 4. Health persons from non-endemic areas (n=10). Both individual's groups 3 and 4 were used as negative controls. All steps were performed at the Damascus University laboratories. We used the

promastigotes from patient *Leishmania* lesions obtained by mass cultivation in RPMI-1640 medium containing 10% fetal calf serum, as the antigens for the serological test. The promastigotes (2×10^6 parasites/ml) from up to 100 ml of stationary-phase cultures were harvested and washed 3 times in phosphate-buffered saline (PBS) (pH=7.4) by centrifugation (5000 rpm, 10 min. +4°C) in order to eliminate the culture media. After measuring the parasite pellet, it was re-suspended in 10% sodium dodecyl sulfate (SDS) (V:V) for 4 minutes and boiled in bath water for 4 minutes then added the sample buffer (0.5M Tris-hydrochloride "pH=6.8," 10% glycerol, 10% SDS, 0.1% bromophenol blue, 5% β mercaptoethanol) in order to obtain approximately 200×10^6 parasite/ml ($5 \mu\text{g}/10^6$ parasite),¹⁴ and boiled as described above. The tube containing proteins was divided into aliquots and stored at -20°C up to the time of use.

The ELISA was applied for the first time in Syria by using the leishmaniasis immunoglobulin G (IgG) CELISA (Cellabs Ltd), which help in detection of the specific antibodies in patient serum samples infected either by cutaneous or by muco-CL.^{7,9} The flat-bottom 96-well microtiter plates were already coated with the CL promastigotes antigen from cultivating *Leishmania mexicana* in protein-free media. The wells were loaded with 100 μl of serum diluted (1:100) in PBS and incubated in humidity at room temperature for 2 hours. The plates were washed 4 times with PBS-T₂₀ and incubated with peroxidase conjugated goat anti-human IgG diluted (1:1000) in PBS in humidity at room temperature for 30 minutes. After the plates were washed 4 times, they were incubated with the substrate 3-ethylbinzthiazolinesulfonic acids, in obscurity for 20 minutes at room temperature, then the reaction was stopped by phosphoric acid (1 M) and the optical density was measured at 450 nm by ELISA reader (Digiscan). The cutoff point was set as optical density (OD)=0.2 nm.

The SDS-polyacrylamide gel electrophoreses (SDS-PAGE) was performed according to the protocol described previously by Schagger and von Jagow.¹⁵ Antigen concentrations ranging from 50-300 $\mu\text{g}/\text{mini-gel}$ and polyacrylamide concentration (12%) were used. The antigens were electrophoresed using mini-protean II electrophoresis cells (Bio-Rad). Each gel included pre-stained protein molecular weight marker (10-250 KDa, Bio-Rad). The gel was then stained with Coomassie brilliant blue for proteins at RT° for one hour. The relative molecular weight of *Leishmania* electrophoresed protein fraction was determined. The gel was also mounted in an antigen transfer apparatus (mini-transfer Blot electrophoretic transfer cell, Bio-Rad) in order to transfer the proteins to the nitrocellulose membranes

(0.22 μm, Bio-Rad) in transfer buffer for one hour at 100 volt. The nitrocellulose membrane was cut into 2-3 mm strips, which were blocked with 5% skimmed milk in PBS for 75 min. Then the strips were washed with PBS-T₂₀ (5 minutes 4 times each), followed by incubation with sera diluted 1:25 in PBS for 75 minutes. After incubation with the primary antibodies, the strips were washed as described above and incubated with goat anti-human IgG, alkaline phosphatase conjugate (Sigma) diluted 1:3000 in PBS for 75 minutes and washed 4 times with PBS-T₂₀. All incubations were performed at RT⁰ on a rotatory shaker. Antibody reactivity was visualized with 5-bromo 4-chloro 3-indolyl phosphate and nitro blue tetrazolium substrate (Sigma).

For determination the statistical significance between the clinical characteristic, both the ELISA and the WB testes results were compared by using Z test for differences in 2 proportions, with 95% confidence intervals ($p=0.01$). Data were analyzed with the SPSS statistical software version 2006.

Results. Our data present that the papular (squamous, lupoid, or erythematous) lesions were found to be predominant in comparison with nodular and ulcerative lesions. There were 168 males (62.2%) and 102 females (37.8%) aged between 10-60 years old that manifested CL lesions. Most of these patients had more than one lesion (average 1-5 lesions) on their upper extremities or face and lower extremities. Two hundred fifty (92.5%)

of the 270 CL (confirmed and suspected) were found to be sero-positive by ELISA test. The absorbance values for the CL patient sera were varied from >0.2-1.0 nm and up, depending on the lesion number and their age (Tables 1 & 2). The significantly higher absorbance value was detected for the patients, who had more than one lesion. A high agreement between the lesion number and the absorbance values were detected ($p=0.01$, Table 3), whereas, no correlations between the lesion age and the absorbance values were detected. The soluble protein profiles of the *Leishmania* parasites obtained by SDS-PAGE showed at least 20 major protein bands with the relative molecular masses ranging from 10-250 kDa.

The sera of 254/270 patients with CL (94%, proved

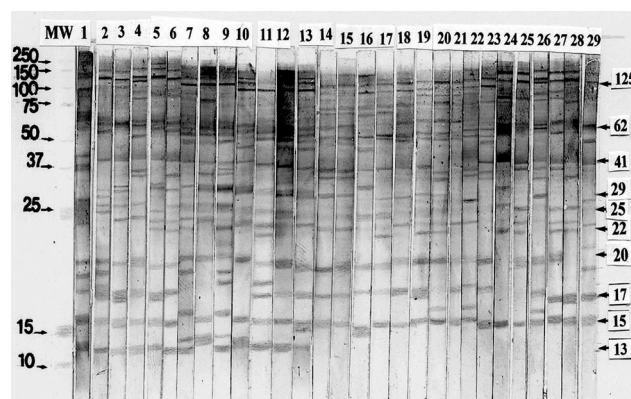


Figure 1 - Western blot results using *Leishmania* parasite soluble proteins and serum from proved cutaneous leishmaniasis (CL) patients (lanes 2-13), suspected CL patients (lanes 14-29). Molecular weight (MW), positive control (lane 1).

Table 1 - Relation between the cutaneous leishmaniasis antibodies rate measured by Enzyme Linked Immunosorbent Assay (ELISA) test and the lesion age.

Lesion age	ELISA test	
	OD >0.2 - 0.5 nm (%)	OD >0.5 - 1.0 ≤ nm (%)
1-6 month (n=219)	133 (60.7)	86 (39.3)
More than 6 month (n=31)	20 (64.5)	11 (35.5)

OD - optical density

Table 2 - Relation between the cutaneous leishmaniasis antibodies rate measured by Enzyme Linked Immunosorbent Assay (ELISA) test and the lesion number.

Lesion number	ELISA test	
	OD >0.2 - 0.5 nm (%)	OD >0.5 - ≤1.0nm (%)
1-5 lesions (n=180)	137 (76.1)	43 (23.9)
More than 5 lesions (n=70)	16 (22.8)	54 (77.1)

OD - optical density

Table 3 - Correlation between Enzyme Linked Immunosorbent Assay test and cutaneous leishmaniasis lesion age and number.

Variables	Lesion number	OD
Lesion age	0.065	0.104
<i>P</i> value	0.286	0.089
Lesion number	-	0.825*
<i>P</i> value		0.000

*Significant correlation at 99% confidence interval ($p=0.01$), OD - optical density

Table 4 - The percentage differences between the Enzyme Linked Immunosorbent Assay (ELISA) and Western Blot (WB) based on Z test.

Test	N	Percentage of positive cases	Standard error of proportion	Percentage difference	<i>P</i> value
ELISA	250	92.5	0.01		
WB	254	94	0.014	1.5	0.49

and suspected), were reacted with several antigens, resulting in multiple bands (Figure 1). The most frequently recognized bands were: 13 kDa (86.6%); 15-17 kDa (92.2%); 20-22 kDa (74.1%); 25-29 kDa (73.3%); 41 kDa (90.7%); 62 kDa (88.5%) and 125 kDa (97%). There was a correlation between the immunoblot patterns and the clinical characteristic of CL disease, such as the number of lesions. Among all antigenic proteins, 7 proteins of 13; 15-17; 20-22; 25-29; 41; 62-66 and 125 kDa bands were detected more frequently in most of CL patients with 5 lesions and more ($OD=0.6 \leq nm$). Whereas, 3 or more proteins (13 or 15-17 in addition to 2 or more) were detected with less than 5 lesions ($OD=>0.2-0.5 nm$). The WB results for CL patients and the negative controls were almost found in agreement with the ELISA results, and there is no significant difference at 95% confidence interval (Table 4).

Discussion. The CL is highly endemic in Syria. The inhabitants have known the disease since longtime, and lesions with different types of clinical characteristics are often seen. The use of serological assays for the diagnosis of CL is very limited due to the easy and cheaper direct diagnosis.¹ To determine the validity of both the ELISA (using leishmaniasis IgG CELISA; Cellabs; Ltd) and the WB (using crude soluble antigen) serological tests used for diagnosis, we carried out a study using CL patients sera obtained from different regions in Syria. The humoral immune response against CL occurs only during the active phase of infection, with the appearance of low titers of antibodies that disappear some months after the end of treatment.^{11,12,16,17} Our finding showed that the humoral response has been changed according to the number of lesions. A positive correlation between the applied serological test results, and the number of lesions was detected. In the ELISA assay, the sero-positivity ratio was calculated to be 92.5%. The absorbance values for the sera of CL patients were significantly higher than the absorbance values for the sera of healthy individuals. A positive correlation between the ELISA results, and the number of lesions in the patient was detected. Our results are in agreement with other studies.^{10,17-20} A comparison of the molecular weight values of protein fractions is a complex process, and many factors such as the parasite growth phase, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel size, the polyacrylamide concentration, and others influence the fraction migration and determination of the molecular weight values.^{6,17,21,22} Therefore, comparison of molecular weights of antigens cited in many studies is always difficult and must be carried out with caution. Bands between 13 and 125 kDa were detected in almost serum samples from the CL patients.

The protein fractions that most reactive with these serum samples have relatively low molecular weights (13; 15-17; 20-22; 29; and 41 kDa). This is similar to the results found for different *Leishmania* parasites.²²⁻²⁴ Isaza et al¹² and Zeyrek et al¹⁷ mentioned that when they used either *L. panamensis* or *L. infantum* Mon1 that the reactivity of sera from CL patients with 120 kDa protein varied between 76.7% and 85.5%. However, the 125 kDa fraction was detected in 97% of the CL patients in the present study. Goncalves et al²² reported that bands between 13 and 150 kDa were detected when they used different *Leishmania* species as the antigen source. Dos Santos et al²⁵ reported on the importance of the 32 and 35 kDa antigenic bands. It is possible that slight modifications in the WB technique could result in the different migrations of diagnostic bands. Therefore, it is possible that 15-17; 20-22; 25-29; 41 and 62 kDa bands correspond to the diagnostic bands identified by many researchers.^{17,26-28} In the present study, proteins 13; 15-17; 20-22; 25-29; 41 and 62 kDa were found to be useful for serodiagnosis.

The parasitological methods were considered as a first choice procedures of the diagnosis of CL due to their high specificity, although their sensitivity may vary depending on the experience of the diagnostic team, who takes the samples and checks the smears, as well as the amount of parasites in the lesion aspirate. However, in some instances, it is very difficult to demonstrate the presence of parasites. In these patients, immunodiagnosis becomes an important alternative for demonstrating the presence of the parasites.^{17,29}

In conclusion, our data suggest that ELISA and WB assays are easy to perform and can be used for the diagnosis of CL in Syria, when the parasites cannot be detected by direct techniques, although the clinical feature of the disease is compatible with leishmaniasis.

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