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***Leishmania braziliensis*-GPI Anchored Membrane Proteins as an Alternative Tool for Specific Sero-Diagnosis of Active American Cutaneous Leishmaniasis**

Agustina Rojas¹, Gladys Crisante¹, Pablo García², José Carrero³,
Néstor Añez^{1,*}

¹Department of Biology, Faculty of Sciences, Centre for Parasitological Research “J. F. Torrealba”, University of Los Andes, Merida, Venezuela

²Laboratory of Biotechnology of Microorganisms (BIOMI), Faculty of Sciences, University of Los Andes, Merida, Venezuela

³Ministry of Health, Dermatological Institute, Tovar-Merida, Venezuela

Email address

nanes@ula.ve (N. Añez)

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Abstract

Purified *Leishmania braziliensis*-GPI-anchored membrane proteins (*L. braziliensis*-GPI-AMP) were antigenically validated to be proposed as candidate antigens to serologically diagnose American cutaneous leishmaniasis (ACL). Validation was carried out in 140 sera-samples, 71 from patients suffering active ACL previously diagnosed using clinical, parasitological, serological and molecular (Polymerase chain reaction-PCR assay) methods, and 69 from individuals either healthy or suffering from other etiologies in order to estimate its sensitivity and specificity, respectively. The use of the *L. braziliensis*-GPI-AMP antigen in western blot assays revealed 99% (70/71) sensitivity to detect circulating anti-*Leishmania* antibodies in sera-samples of the tested patients. The positive reactivity was evidenced by a constant band of 50 kDa molecular mass detected in all the patients suffering ACL. A 100% specificity of the *L. braziliensis*-GPI-AMP antigen as diagnostic tool was recorded after confirming negative results in all control samples. The obtained values of sensitivity (99%), specificity (100%), positive predictive value (1.00) and negative predictive value (0.99) demonstrated the reliability of the *L. braziliensis*-GPI-AMP antigen to sero-diagnose ACL. In addition, the constant expression of the 50kDa band, led us to consider it as a biochemical marker capable of producing reliable, sensitive and specific signal to detect active ACL.

1. Introduction

The serological diagnosis of American cutaneous leishmaniasis (ACL) has been considered a very difficult task involving the technique itself as well as the problems related to the particular immune-humoral response of infected individuals. Regarding the serological tests for diagnosis of ACL most of them are based on crude antigens or not purified fractions prepared from cultured promastigotes forms, which are far from reliable. In this case, tests frequently show cross reaction with other species of the genus *Leishmania*,

or even with parasites of the family Trypanosomatidae, making diagnosis inconsistent and inadequate to its purpose (1, 2, 3). In relation to the humoral response of patients suffering ACL, some authors have reported that anti-*Leishmania* antibodies circulate in scarce amounts, which are not recognized by conventional serological tests (4, 5). This fact has been confirmed using conventional techniques including, among others, direct agglutination test (DAT), immunofluorescence antibody test (IFAT) and enzyme-linked immuno-sorbent assay (ELISA) to detect anti-*Leishmania* antibodies in ACL-patients in different clinical conditions (3). This way, the used conventional tests revealed relatively low seropositive values compared with methods such as leishmanin-skin test (LST), PCR assay or detection of the parasite itself in active lesions, suggesting that conventional serological tests are unreliable to diagnose ACL (3). Trying to propose better techniques able to produce reliable and reproducible serodiagnosis for ACL as those used to diagnose visceral leishmaniasis (6) a variety of molecules considered as specific proteins from the *Leishmania* surface have been assayed. These include, among others, glycosyl-phosphatidyl-inositol (GPI) anchored membrane proteins (AMP) isolated and purified from *L. braziliensis* in enough amounts to be antigenically evaluated (7). Consequently, *L. braziliensis*-GPI-AMP should show particular characteristics as antigen to be specifically recognized by antibodies generated by the immune system of individuals suffering ACL (8). The present article deals with the use of the *L. braziliensis*-GPI-AMP purified antigen as a tool to specifically diagnose ACL in sera samples from patients bearing an active *Leishmania*-infection.

2. Patients and Methods

2.1. Study Groups

A total of 140 individuals from 34 localities belonging to 6 federal entities of western Venezuela were selected to carry out the present study. They were organized into two groups. Group I was made up of 71 patients suffering active American cutaneous leishmaniasis (ACL) previously diagnosed using different methods. These included: i. parasitological techniques such as Giemsa stained smear from lesions and/or cultured parasites, ii. Leishmanin-skin test (LST) to detect the host cellular immune-response, iii. conventional serological tests (DAT, IFAT, and ELISA) and Polymerase Chain Reaction (PCR) assays. Details on the procedure of the methods used have been provided in a previous report (3). This group was composed of 40 males (56%) and 31 females (44%) with a 1.2:0.8 male: female ratio, showing an average age of 32 years $\pm$ 21 years with a range of 1-59 years. Group II was made up of 69 individuals from different geographical areas and clinical conditions; 45 of them were either healthy control (15) or patients suffering from etiologies other than ACL (30). The remaining 24 were patients who showed evidence that they had suffered cutaneous leishmaniasis but cured after

receiving etiological treatment.

2.2. Parasites

Promastigote forms of *Leishmania (Viannia) braziliensis*, isolate MHOM/Ve/75/LMR-75, kept in NNN culture medium were selected to carry out the present work. Prior to the study, identification of the selected isolate was confirmed by PCR assay using primers for the spliced leader RNA (mini-exon) gene sequence: LU-5A 5' TTT ATT GGT ATG CGA AAC TTC 3'; LB-3C- 5' CGT SCC GAA CCC CGT GTC 3'; LM-3A- 5' GCA CCG CAC CGG RCC AC 3' and LC-3L- 5' GCC CGC GYG TCA CCA CCA T 3', following methodology previously reported (9). The selected isolate of the parasite was kept at 25°C until reached masses of flagellates at the exponential phase equivalent to 8 X10⁸ promastigotes/mL approximately. The flagellates were collected and centrifuged at 3000Xg at 4° C for 15 min, washed with phosphate buffered saline (PBS) pH 7.2, and maintained at -20°C until use.

2.3. Isolation Purification and Use of *L. braziliensis*-GPI Anchored Membrane Proteins as Antigens in the Diagnosis of American Cutaneous Leishmaniasis

GPI- anchored membrane proteins from the selected isolate of *L. braziliensis* was obtained using the partition Triton-X114 method previously described (10) and later adapted to *Leishmania* in our laboratory (7). With this methodology we isolated and purified *L. braziliensis*-GPI-AMP, being then separated in 12% SDS-PAGE and electro-transferred into Immobilon P membrane in order to be used as antigen to perform western blot assays. For each of the ACL patient to be diagnosed with the western blot method, the primary antibody consisted of an aliquot of anti-*Leishmania* serum diluted 1:100 and as secondary antibody, dilutions of human anti-IgM (1:6000), anti-IgG (1:8000) and polyvalent (1:5000 anti- IgM, IgG, IgA) conjugates associated to peroxidase. Details of the isolation and purification of GPI-AMPs as well as those for the validation of antigenicity using the western blot assay are found in protocols previously reported by our research team (7, 11).

2.4. Diagnostic Criteria

The individuals selected to carry out the confirmatory sero-diagnosis protocol using the here proposed *L. braziliensis*-GPI-AMP antigen, were previously diagnosed in order to ensure that the studied samples came from unquestionable patients bearing active ACL. To fulfill this condition, several methods and diagnostic criteria were done as follows: i. microscopically visualization of *Leishmania* amastigotes in tissues smeared on glass slides stained with Giemsa; ii. Positive leishmanin skin test (LST) consisting of indurations $\geq$ 5 mm in diameter 48 hours after injection; iii. Detection of specific *L. braziliensis* DNA in tissue samples from skin lesions using PCR assays; iv. Seropositive results using conventional serological techniques (DAT, IFAT, ELISA)

recognized by showing reactivity in at least 2 of the 3 sero-tests. In addition, sera samples from the selected patients with active ACL to be diagnosed using the *L. braziliensis*-GPI-AMP antigen were declared positive when western blot revealed immunologic activity expressed as conspicuous bands of about 50 kDa.

2.5. Statistical Analysis

The confirmatory diagnosis of the *L. braziliensis*-GPI-AMP as antigen was established comparing it with conventional serological tests (DAT, IFAT and ELISA) and PCR assays. Statistical analysis was carried out estimating parameters such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), the statistic Z and P value, with 95% confidence intervals (12, 13).

2.6. Ethical Considerations

A written consent including the agreement prior information (API forms) from all the patients who entered the protocol was obtained before the sample collection started in order to comply with the criteria established by the Biomedical Committee of the National Research Council of Venezuela.

3. Results

3.1. Validation of the *L. braziliensis*-GPI-AMP Antigen for the Specific Sero-Diagnosis of Active American Cutaneous Leishmaniasis

The validation of the *L. braziliensis*-GPI-AMP antigen, proposed to be used as an alternative reliable test to diagnose active ACL, was carried out using 140 sera samples from

patients with different clinical conditions. From the selected sera samples, 71 (51%) of them were taken from unquestionable patients suffering active ACL previously confirmed by various diagnostic methods as indicated (Fig. 1). The samples were comparatively analyzed with conventional serological techniques (DAT, IFAT, ELISA) and PCR assays in order to assess the sensitivity of *L. braziliensis*-GPI-AMP as alternative diagnostic tool. The use of the *L. braziliensis*-GPI-AMP antigen in western blot assays revealed 99% sensitivity to detect circulating anti-*Leishmania* antibodies in samples from the tested patients (Table 1). The positive reactivity of sera from patients suffering active ACL to *L. braziliensis*-GPI-AMP antigen was evidenced by the presence of constant bands of 50 kDa (Fig. 2). The sensitivity of the *L. braziliensis*-GPI-AMP antigen, used as diagnostic tool, showed higher values when compared with conventional serological techniques, being the difference statistically significant ($P < 0.001$). On the other hand, comparison between PCR assay and the here proposed diagnostic tool showed no significant difference ($P > 0.05$). In addition, the remaining 69 sera samples comprising those from 24 individuals who had suffered and cured cutaneous leishmaniasis and 45 from patients with other etiologies, showed negative results in all cases, demonstrating 100% specificity of the *L. braziliensis*-GPI-AMP antigen as diagnostic tool (Table 2). Considering the specificity of this protocol, the statistical comparison revealed no significant difference ($P > 0.05$) among the three used tests, indicating that they are able to equally recognize negative samples. Details on the statistical comparison among the analyzed variables used to validate the *L. braziliensis*-GPI-AMP antigen as reliable test to diagnose active ACL, including sensitivity, specificity, positive predictive value, negative predictive value, the statistic Z and p value, are given in Table 3.

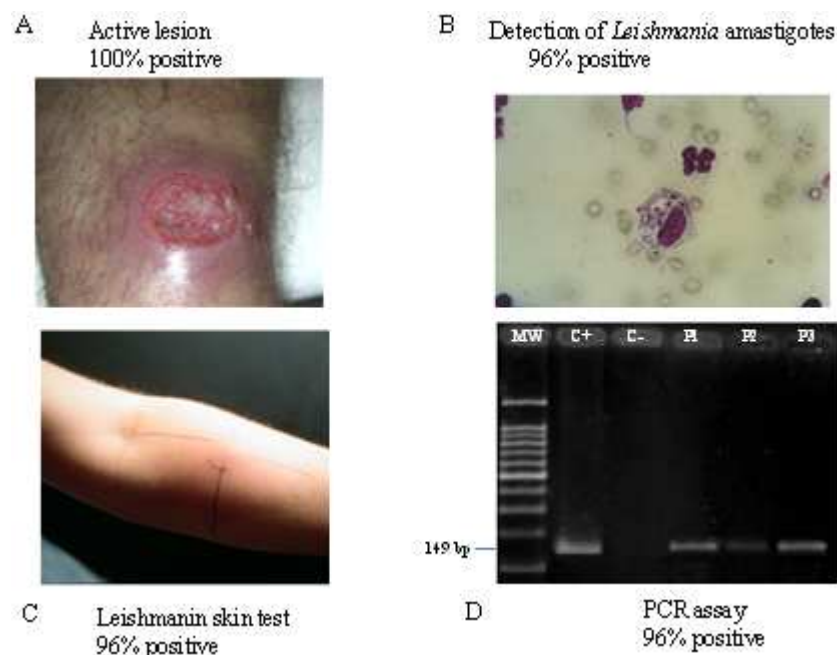


Figure 1. Characteristic of selected patients showing active ACL to validate *L. braziliensis*-GPI-AMP as sero-diagnostic test. Note active lesion (A), *Leishmania* amastigotes from a lesion in stained sample (B), positive leishmanin skin test (C) and positive PCR assay (D).

Table 1. Sensitivity of the *L. braziliensis*-GPI-AMP antigen as compared to conventional serology and PCR assay.

Conventional serology	PCR assay	<i>L. braziliensis</i> -GPI-AMP
N° (%) +ve N°(%) -ve	N° (%) +ve N°(%) -ve	N° (%) + N°(%) -ve
5 (7)* 66 (93)	68 (96)* 3 (4)	70 (99)* 1 (1)

*: Comparative sensitivity

Table 2. Specificity of the *L. braziliensis*-GPI-AMP antigen validated with control sera samples from different sources.

Sample from Patients with	Number	Detected serological results using		
		Conventional serology	PCR assay	<i>L. braziliensis</i> -GPI-AMP antigen in Western blot
Visceral leishmaniasis	5	Negative	Negative	Negative
Cured cutaneous leishmaniasis	20	1 Positive 19 Negative	Negative	Negative
Cured muco-cutaneous leishmaniasis	4	Negative	Negative	Negative
Negative from endemic area	5	Negative	Negative	Negative
Negative from non-endemic area	10	Negative	Negative	Negative
Varicose ulcer	4	Negative	Negative	Negative
Acute Chagas disease	5	Negative	Negative	Negative
Chronic Chagas disease	5	Negative	Negative	Negative
<i>Trypanosoma rangeli</i> infection	5	Negative	Negative	Negative
Mosquito bites ulcer	3	Negative	Negative	Negative
Post Hamsen ulcer	1	Negative	Negative	Negative
Traumatic ulcer	1	Negative	Negative	Negative
Cancer ulcer	1	Negative	Negative	Negative
Sources: 13	69	68/69	69/69	69/69
Specificity (%)		99%	100%	100%

Table 3. Statistical comparison among tests to diagnose active ACL.

Variable	Conventional serology value	PCR assay value	<i>L. braziliensis</i> -GPI-AMP value
Sensitivity (95% CI)	0.11(0.04-0.19)	0.93(0.87-0.99)	0.99(0.96-1.00)
Specificity (95% CI)	0.99(0.96-1.00)	1.00(1.00-1.00)	1.00(1.00-1.00)
Positive predictive value (95% CI)	0.89(0.68-1.00)	1.00(1.00-1.00)	1.00(1.00-1.00)
Negative predictive value (95% CI)	0.52(0.43-0.60)	0.93(0.88-0.99)	0.99(0.96-1.00)
Z		-9.78	-1.82
P value		0.001	0.034

3.2. Detected Immuno-Reactivity Patterns in Sera from Patients with Active ACL Using *L. braziliensis*-GPI-AMP Antigen in Western Blot

From the 70 positive sera samples of ACL patients diagnosed with the western blot method using the *L. braziliensis*-GPI-AMP as antigen, three reactivity patterns were recognized according to the migration of the expressed bands, being constantly recognized in all of them a 50 kDa band. The analysis distinguished a major pattern observed in 50 (71%) sera samples, in which only a single band of 50 kDa was observed when the western blot was developed with the secondary antibodies (Fig. 3A). In addition, a second pattern recognized by the expression of two protein bands of 50 kDa and 28 kDa in 16 (23%) of the studied patients was also detected (Fig. 3B). Finally, a third minor immuno-reactivity pattern was detected in 4 (6%) samples in which several bands ranging from 20 kDa to >100 kDa were recognized in western blots developed with the polyvalent anti-human secondary antibodies (IgA, IgM, IgG) as shown in Fig. 3C.

4. Discussion

In the present study *L. braziliensis*-GPI-AMP fractions

were antigenically evaluated using sera from patients suffering unquestionable active cutaneous leishmaniasis previously diagnosed clinically (presence of lesion), parasitologically (detection of *Leishmania*-amastigotes), immunologically (LST with indurations >5mm in diameter) and molecularly (positive PCR assays). The use of these protein fractions as antigens along with the circulating anti-*Leishmania* antibodies generated in the selected ACL patients to carry out the chosen western blot technique, revealed reactivity in 70 of the 71 (99%) selected sera samples. This fact seems to indicate that *L. braziliensis*-GPI-AMP should be a potential tool to sero-diagnose patients suffering active ACL. Indeed, the fact that a peptide of 50 kDa was constantly revealed in all the 70 sera-samples of the recorded infected patients pointed out that this purified antigen may be considered as a biochemical marker to be used in the diagnosis of active ACL. To demonstrate this potentiality, we compared the results obtained using the *L. braziliensis*-GPI-AMP purified antigen with those detected when conventional serology and PCR assays were used. This way, we detected a significant difference ($P < 0.001$) between reactivity observed when using serological techniques (DAT, IFAT, ELISA), which revealed 7% sensitivity, and the 99% obtained with the *L. braziliensis*-GPI-AMP antigen. This result is particularly important in order to demonstrate that the use of crude or not

purified antigens is not effective enough to sero-diagnose ACL, supporting previous observations (3). Conversely, the comparison with PCR results revealed no significant difference ($P>0.05$) indicating that the use of the here proposed biochemical marker as a tool for sero-diagnosis is as good as the method to detect *Leishmania* DNA (9, 14). In addition, the validation of the specificity of *L. braziliensis*-GPI-AMP fractions revealed high reliability demonstrated by its capability for distinguishing all negative controls and, which appears to be more important, because it did not cross-react with sera from patients with visceral leishmaniasis, cured tegumentary leishmaniasis, Chagas disease, *T. rangeli* infection, as well as with sera from individuals with other etiologies. These results also support previous experimental observations in which no reactivity was detected when GPI-AMP fractions of *L. mexicana* and *L. amazonensis* were exposed to *L. braziliensis*-GPI-AMP rabbit immune-sera (7). Taking together the high levels of sensitivity (99%), specificity (100%), positive predictive value (1.00)

and negative predictive value (0.99) obtained during the validation of the *L. braziliensis*-GPI-AMP as antigen to sero-diagnose ACL, allow us to consider the 50 kDa protein fraction as a candidate to produce a reliable, sensitive, specific, fast and economical method to detect active ACL. The above results obtained with the here showed methodology are supported by recent findings using *Trypanosoma cruzi*-GPI-AMP to sero-diagnose patients suffering Chagas disease infection (15). In this case, antigenic protein fractions ranging from 23 kDa to 123 kDa were specifically recognized by anti-*T. cruzi* antibodies in sera from acute and chronic chagasic patients, showing values of sensitivity and specificity similar to the ones revealed in the present work. Interestingly, our results using the *L. braziliensis*-GPI-AMP antigen appears to have some advantages over diagnostic techniques such as LST and PCR assays because the latter are not capable to discriminate between active and cured cases of leishmaniasis as former does.

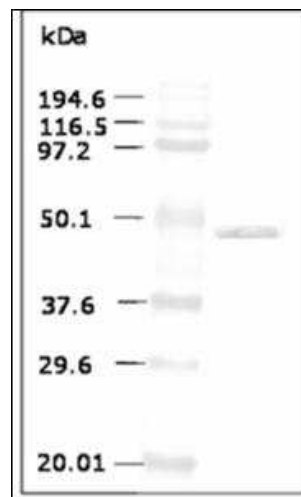


Figure 2. Western blot showing a 50 kDa positive band reaction to *L. braziliensis*-GPI-AMP against a serum from a selected patient suffering active ACL.

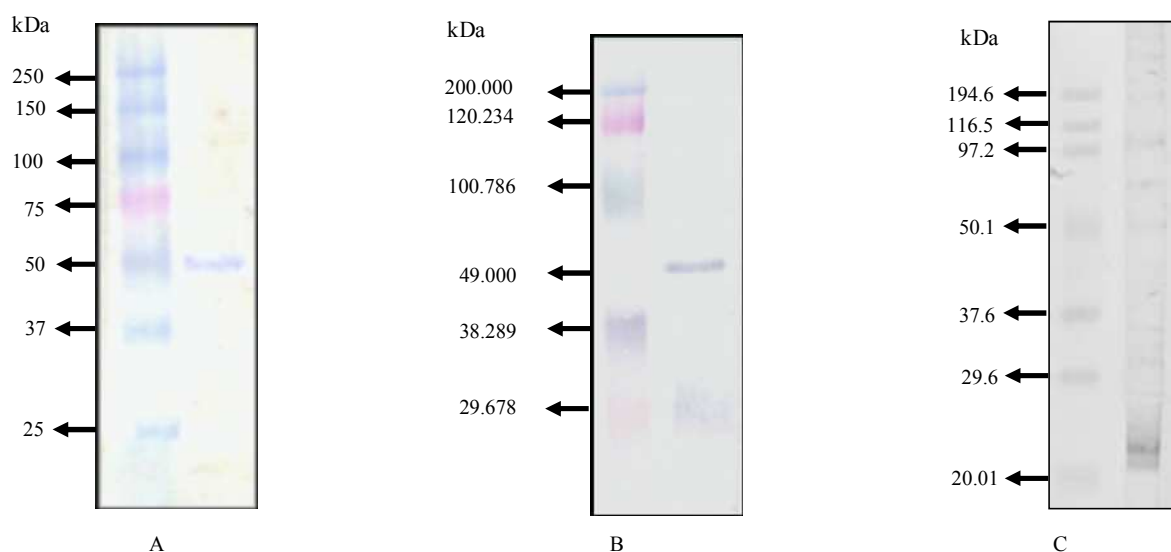


Figure 3. Observed patterns in sera from patients with active ACL using *L. braziliensis*-GPI-AMP antigen in western blot assays. Note the reactivity showing bands of A. 50 kDa; B. 28 kDa and 50 kDa; C. 20 kDa to over 100 kDa as explained in the text.

Regarding the observed immuno-reactivity patterns in sera from patients with active ACL using the *L. braziliensis*-GPI-AMP antigen in a western blot method, developing each sample with human anti-IgM, anti-IgG and polyvalent (anti- IgM, IgG, IgA) conjugates as secondary antibodies, it is of interest to note the constant expression of a band of 50 kDa in the 99% positive samples, confirming the active ACL infection irrespective of the evolution or characteristic of the cutaneous lesion. Nonetheless, three reactivity patterns were detected in the patient's sera when western blots were developed using IgM, IgG and polyvalent conjugates as secondary antibodies. Indeed, in 50 patients (71%) the analysis revealed a sole reactive band of 50 kDa, whose intensity was independent of anti-sera titer, clinical manifestation, lesion evolution time and geographical origin of patients, supporting similar results for previous tested *L. braziliensis* peptide (16). A second pattern was detected in sera-samples from 16 patients (23%) showing a pair of bands in the range of 28 kDa and 50 kDa, and a third pattern expressing multiple bands ranging from 20 kDa to over 100 kDa, but conserving reactivity as a 50 kDa band as previously mentioned. In relation to the observed pattern, we have not yet argument to explain the expression of different immuno-reactivity to the *L. braziliensis*-GPI-AMP antigen, since in leishmaniasis it is very difficult to obtain a completely homogeneous antibody. This is particularly true due to the pathology in *Leishmania*-infected host depends on the strength of cell mediated immunity and its balance with humoral response. Consequently, more investigations are suggested in order to correlate the reactivity patterns with variables such as time of evolution of the infection, *Leishmania*-isolates, parasite load, and origin of patients.

5. Conclusion

The high sensitivity and specificity revealed during the validation of *L. braziliensis*-GPI-AMP as a purified antigen to sero-diagnose ACL and the constant detection of a 50 kDa band, led us to conclude that this protein fraction may be proposed as a biochemical marker to produce a sensitive, specific and fast tool to detect active ACL. In addition, the fact that it does not cross-react with sera from patients infected with visceral leishmaniasis, Chagas disease or other etiologies makes this peptide highly reliable allowing us to strongly recommend it as an alternative tool for specific diagnosis of ACL.

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