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Evaluation of serological assays based on a novel excreted antigen preparation for the diagnosis of Cutaneous Leishmaniasis in Panama☆

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Abstract

The objective of the present study was to determine the efficacy of prototype diagnostic serological assays for American Cutaneous Leishmaniasis (ACL) in Panama. As such, we prospectively sampled 100 cutaneous leishmaniasis case-patients and tested their sera in two serological assays based upon novel soluble antigen preparations made from propagating the parasites in a protein-free, serum free media. Using serum and a *Leishmania mexicana* antigen preparation to sensitize plates, the assay correctly identified 89% of the case-patients. While using serum with an antigen preparation from *Leishmania braziliensis*, the assay correctly identified 71% of the patients. Concerning both test formats, performance was near equal in true positive and presumptive positive subsets demonstrating the improved sensitivity of these assays over reference methods of choice. Since the incidence of leishmaniasis in Panama has increased dramatically in the past 10 years, these assays may be useful in clinical and epidemiological studies and control programs.

Keywords: Diagnosis; Serology; Cutaneous leishmaniasis; Panama

1. Introduction

The leishmaniases constitute a wide spectrum of disease manifestations in man. They occur when a susceptible host is bitten by a Phlebotomine sandfly infected with *Leishmania* parasites. Leishmaniasis

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may develop as a visceral, mucocutaneous or cutaneous disease. The cutaneous form of the disease is most common with an estimated 1.5 million new cases per year worldwide (Berman, 1997). American Cutaneous Leishmaniasis (ACL) occurs from southern Texas to northern Argentina. Throughout the world one may encounter sporadic cases of relatively insignificant public health importance to hyperendemic regions like northern Afghanistan where almost all individuals suffer from the disease by the age of 12. Cutaneous leishmaniasis (CL) is generally most feared in Latin America because of its association with mucocutaneous leishmaniasis, a chronic destructive condition that is difficult to treat. However, in many hyperendemic areas there is a public acceptance for the condition where many infected will not seek treatment.

The causative agent of ACL was first reported in 1909 in Brazil. One year later, Darling documented the first case of autochthonous *Leishmania* transmission in Panama (Sanchez et al., 1992). Since that time, there has been a long history of case reporting, epidemiology and disease ecology studies throughout Latin America and Panama. Members of both the *Leishmania viannia* and *mexicana* complexes are found in Panama (Kern and Pedersen, 1973; de Vasquez et al., 1990). In Panama, two-toed sloths are the primary reservoir for *Leishmania panamensis* (Herrer and Christensen, 1980). However, forest rodents are the primary reservoirs for many of the *L. mexicana* complex members (Herrer et al., 1973).

Attempts at disease intervention are stifled by the absence of a technique that accurately and practically diagnoses ACL suspects. In terms of the way diagnosis is performed in most clinics today, little has changed in nearly a century. Parasites must be demonstrated from a biopsy of infected tissue for confirmatory diagnosis. Speciation can only be done after expansion of parasite numbers in culture. One new assay, the rK39 dipstick, is a very specific diagnostic for active kala azar, but has not been shown to be useful in the diagnosis of CL. In addition, there are no other reliable antigens for the development of immunodiagnostic tests for diseases caused by the other *Leishmania* species. Even though several DNA primers exist for *Leishmania* detection, none have been consistent enough to gain widespread use. A dire need persists for the development of practical sensitive and specific tests.

Often, new diagnostics measure a patient's antibody response and use it as a correlate for infection or exposure. Serological tests for diagnosing visceral leishmaniasis (VL) were shown to be highly sensitive (Senaldi et al., 1996; Bagchi et al., 1998). However, serological tests are rarely performed to diagnose cutaneous leishmaniasis. Previous attempts reported performance parameters that were disappointingly low (Anthony et al., 1980; Garcia-Miss et al., 1990). Antigens used as the basis for previously reported tests originated from cultured promastigotes (Choudhary et al., 1990, 1992; Badaro et al., 1986) or recombinant proteins (Reed et al., 1990). However, crude antigen preparations lack the metabolic products that promastigotes release into their environment. These metabolic products should be included in any diagnostic strategy since their immunogenicity is well established (Schnur et al., 1972; Sergeiev and Shikuna, 1969). Excreted factor, a component of these antigens, is a negatively charged carbohydrate-like substance which was shown to precipitate antibody from homologous sera of promastigote-infected rabbits (El-On et al., 1979; Bray and Lainson, 1966).

New antigen preparation techniques involving a protein-free, serum-free media formulation (XOM) led to a soluble antigen preparation from *Leishmania donovani* that was the basis for a very sensitive and specific ELISA for kala azar case-patients (Martin et al., 1998). Recently, we improved this antibody-capture ELISA and demonstrated its ability to detect *Leishmania*-specific immunoglobulin G (IgG) in the sera of patients with cutaneous leishmaniasis. In preliminary studies with 143 ACL patients from Brazil, the assay correctly identified 92.3% of the samples (Ryan et al., 2002).

The purpose of the present study was to evaluate the sensitivity of these tests in Panama by employing standardized methods for patient sampling and specimen processing.

2. Materials and methods

2.1. Population

The study was performed at the Gorgas Memorial Institute, Panama City, Panama under an approved human use protocol. A total of 100 adults presenting

to the Tropical Medicine Clinic with lesions consistent with ACL were solicited for the study. Informed consent was gathered from all subjects that chose to volunteer for the study. A medical doctor and laboratory technician experienced in diagnosis and treatment of ACL contacted and sampled the research subjects. A questionnaire was applied to determine the medical history of the patient and any conflicting etiologies. Each subject in the study was assigned a code number. This code number was attached to any samples taken in the study, thereby protecting the identity of the test subjects.

2.2. Sample processing

Six milliliters of blood were collected from each subject. The tube was centrifuged to separate out the cellular fraction. Sera was decanted and stored at −20 °C until it could be analyzed. For confirmatory diagnosis, we removed small amounts of tissue from the border of the lesion by scraping with a sterile surgical blade. Prior to using the blade, 1% lidocaine hydrochloride was applied subcutaneously to the skin, as anesthetic. When necessary, a lesion sample was obtained by using a 3 mm punch. Tissue was used to inoculate culture medium for propagation of promastigotes and was also applied to the surface of glass microscope slides. The slides were fixed in methanol and left to air-dry. A giemsa stain of the slide was made and the cellular material on the slide examined under high power (100×) with a standard light microscope. An experienced microscopist confirmed the presence or absence of amastigotes in the macrophages of the patient sample.

2.3. Antigen and ELISA plate preparation

Washed promastigotes were inoculated into 200 ml of a defined, conditioned protein-free medium and the inoculum was adjusted with a haemocytometer to give a final density of 1×10^8 cells/ml. The parasites were incubated at 26 °C for 72 h in 2-l roller bottles. Thereafter, the spent medium was harvested by centrifugation at 9000 × *g* for 30 min and the relative protein concentration of the soluble antigens (exo-antigen) was estimated by measuring the optical density at 280 nm (Peterson, 1983). Plate sensitization was effected by coating polystyrene, 96-well microtiter plates (Immulon IV, Dynatech Laboratories, Chantilly, VA) with 0.1 ml of the respective exo-antigen solution (5 μg protein per well). *Leishmania braziliensis* (MERTU1350, Guatemala Strain) and *L. mexicana* (UNAM-FD, Mexico) exo-antigen was used to sensitize plates. Plates were then blocked with 0.5% casein (Sigma, St. Louis, MO) in PBS for 1 h at room temperature.

2.4. Cutaneous leishmaniasis antibody-capture ELISA

A standard enzyme-linked immunosorbent assay (ELISA) for the detection of Leishmania-specific IgG was used to assay patient serum. This ELISA format is intended only for screening host sera for IgG antibody detection. Briefly, the sample was diluted in a 0.5% boiled casein-blocking buffer and absorbed onto the sensitized microtiter plates described above. Following a 2-h incubation period, the plate was washed four times with PBS-Tween. A solution containing an HRP-conjugated anti-human IgG polyclonal antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was applied to the plate for 30 min. The plate was washed four times and a substrate (TMB, K&PL) was applied. The optical density values of the samples were monitored at 650 nm with a plate reader until the value of the positive control reached an optical density of 0.85. Following this, a 0.1 N solution of phosphoric acid solution was applied to stop the enzymatic reaction. The plate was reread at 450 nm and the data analyzed. Study subject samples were compared to a group of known negatives ($n = 12$) and positives ($n = 3$) run on each plate. Basically, the known positives were used as a reference to demonstrate a positive value and the negatives were assayed in triplicate to provide a basis for comparison. The mean value and standard deviation of the negative control group (3 × 's standard deviation + mean) were used to establish a cutoff score (Kurstak, 1985).

3. Results

3.1. Subjects

A total of 100 adult research volunteers presenting to the Tropical Medicine Clinic at the Gorgas Memorial Institute of Health Studies were recruited for this study.

Ages ranged from 18 to 84, with a mean of 36.5. There were 74 males and 26 females in the study group.

3.2. Lesions

Research subjects with classic signs and symptoms of ACL presented with lesions ranging in number from a single small or large lesion to multiple lesions. One subject presented with more than 100 lesions. Minus this one patient, which we considered to be very unusual, the mean number of lesions for the group was 2.57, and a median value of 2.

3.3. Chronicity

In accordance with patient recollection, the duration of their dermal condition was from 3 weeks to 5 months, with an average of 1.86 months. The evolution of the skin lesions in 35 patients was of 1 month or less; in 42 patients was of 2 months; in 14 patients was of 3 months; and, in 7 individuals more than 4 months duration.

3.4. Conventional diagnoses

Only those samples exhibiting well-defined parasitemia by smear and/or culture positive were considered to be true positive. Forty-six patient samples were positive by culture and 55 were positive by smear. Together, 28 were positive for both techniques. We make a distinction here between two groups; those that were positive by smear and/or culture, the true positives; and, those that were not positive by either conventional diagnostic, however, given the experience of the clinician working with ACL, we are certain these patients had the disease and therefore designate this group as presumptive positive. Given these criteria, there were 63 true positives (TP) and 37 presumptive positives (PP) enrolled in this study.

Table 1
Performance of antibody-capture ELISA for ACL based upon a *L. mexicana* antigen preparation versus true positive (TP) and presumptive positive (PP) patient subsets

Serum ELISA LM	TP	PP
Total number of values	63	37
Minimum	0.2185	0.08
25% Percentile	0.5875	0.3965
Median	0.912	0.6035
75% Percentile	1.54725	1.0485
Maximum	4.646	3.0615
Mean	1.216135	0.898351
Standard deviation	0.929557	0.751427
Standard error	0.117113	0.123534
Lower 95% CI	0.982026	0.647811
Upper 95% CI	1.450243	1.148891
Overall accuracy	89%	

Table 2
Performance of antibody-capture ELISA for ACL based upon a *L. braziliensis* antigen preparation versus true positive (TP) and presumptive positive (PP) patient subsets

Serum ELISA LBB	TP	PP
Total number of values	63	37
Minimum	0.1745	0.091
25% Percentile	0.50975	0.372
Median	0.8455	0.565
75% Percentile	1.44975	1.1445
Maximum	2.7115	3.0225
Mean	0.970103	0.881541
Standard deviation	0.632124	0.721375
Standard error	0.07964	0.118593
Lower 95% CI	0.810903	0.641021
Upper 95% CI	1.129303	1.122061
Overall accuracy	71%	

3.5. ELISA

Using serum and a *L. mexicana* antigen preparation to sensitize plates, the assay correctly identified 89%

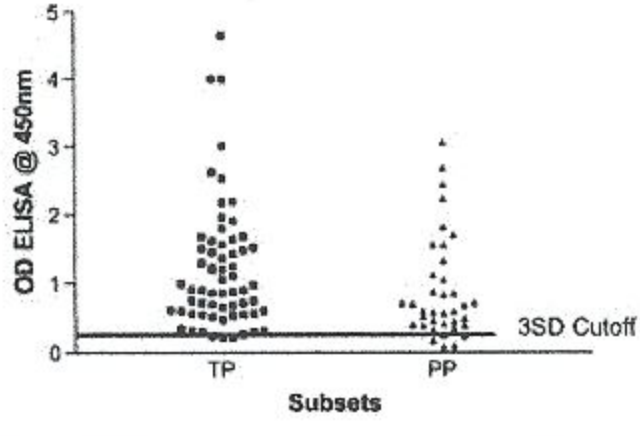

Fig. 1. Scatter plot showing sample optical density values of antibody-capture ELISA for ACL based upon a *L. mexicana* antigen preparation versus true positive (TP, $n=63$) and presumptive positive (PP, $n=37$) patient subsets. Position of negative cutoff line represents the mean plus 3 standard deviations of a group of naïve North American normal control sera ($n=12$).

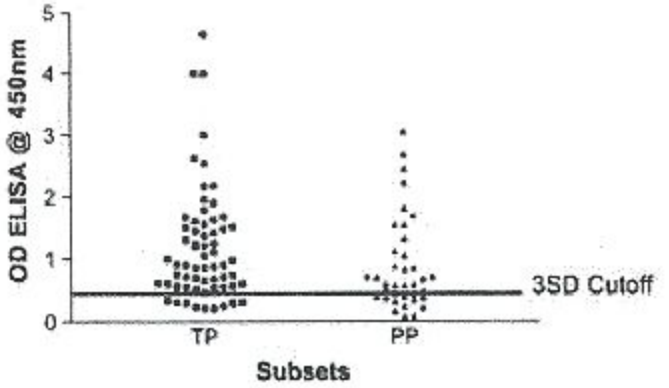

Fig. 2. Scatter plot showing sample optical density values of antibody-capture ELISA for ACL based upon a *L. braziliensis* antigen preparation versus true positive (TP, $n=63$) and presumptive positive (PP, $n=37$) patient subsets.

of the case-patients (Table 1). While using serum with a *L. braziliensis* antigen preparation, the assay correctly identified 71% of the patients (Table 2). Concerning both test formats, performance was near equal in true positive and presumptive positive subsets demonstrating the improved sensitivity of these assays over reference methods of choice (Tables 1 and 2). Figs. 1 and 2 demonstrate graphically by scatter plot the OD ELISA values (at 450 nm) of the patient samples for the two test formats. A negative control group ($n=12$) was used to compute the negative cutoff score (3 SD + mean) for each chart.

4. Discussion

Cutaneous leishmaniasis is endemic in several tropical and subtropical countries around the world, mostly affecting populations that reside in rural, remote areas where basic health services are not available. In this setting, diagnosis is limited to clinical-epidemiological characteristics because confirmatory laboratory tests are available only in reference centers that are inaccessible for most of these patients. The standard methods more often employed to diagnose leishmaniasis are the Montenegro skin test, smear preparations and culture isolation of the parasite. However, each of these traditional methods has several limitations. The Montenegro skin test, which is very simple and highly sensitive, lacks specificity and cannot distinguish between active, inactive or past infection. Direct microscopic identification of the parasite is simple but its low sensitivity of 57% in ACL (Pirmez et al., 1999) is particularly more problematic in chronic cases where parasitemia is low. Cultivation of tissue samples is probably the best parasitological method since it allows the isolation of the parasite in 60% of the cases and allows further species identification, but it is time-consuming and it is subject to microbial contamination. Traditional serological tests aimed at detecting circulating antibodies against the parasite have shown a tendency towards cross-reactivity and low sensitivity presumably because of low antibody titers (Kar, 1995).

The principal objective of the present study was to evaluate a novel ELISA test based on detection of antibodies against leishmania exogenous antigens in 100 Panamanian patients with clinical and epidemiological data compatible with CL and a positive Montenegro test. The results presented here demonstrate that ELISA tests based on antigen preparation from *L. mexicana* (Lm) and from *L. braziliensis* (Lb) performed better than conventional serological methods previously reported (Pirmez et al., 1999). A sensitivity of 89% was obtained using antigen from Lm, and 71% sensitivity employing Lb antigen. This sensitivity is superior when compared to the conventional diagnostic tests performed in the same individuals. In this case, the microscopic identification of the parasite on smears was confirmed in 55% of the cases, whereas parasite isolation was possible only in 46% of the patient tissues. Thus the definitive diagnosis was established in 63% of the cases employing either the microscopic or the parasite isolation techniques and was identified as true positives (TP). While the 37 patients that were not positive by either conventional diagnostic test were designated as presumptive positive (PP). The serologic diagnosis was missed in only 3 of the 63 TP when Lm antigen was used. Whereas 15 TP patients were negative when analyzed by the ELISA prepared with Lb antigen. Similarly, the leishmanial etiology was not established in 5 samples from the 37 PP patients using Lm antigen, and in 14 samples by using Lb antigen. Thus, the relative high positive rate in the TP group by using the Lm antigen suggests that this antigen is far superior to the Lb antigen. The fact that antigens from both leishmania species were prepared under identical technical con-

ditions suggests that the difference in antigenic quality may depend on factors inherent to the particular species of the parasite.

A direct comparison between TP and PP groups through the ELISA profile shows very similar results, suggesting that the clinician was experienced enough to know ACL when he saw it, even though conventional tests failed. As evident from these serological results, the ELISA test measures these two groups nearly equally because it is not subject to the same physical limitations as low parasitemia in chronic lesions and culture contamination. However, this does not exclude the possibility of active or inactive lesions with low or no humoral immune response from the host. In this case, serology could be misleading. On the other hand, since the dynamics of the antibody response in ACL patients is unknown, it is impossible to say what certain levels of IgG detection means other than the patient was exposed to the parasite. An interesting observation in our study is that the three TP patients not detected by this test had early lesions with only 4 weeks of evolution. Whereas the five PP individuals with an ELISA-negative result had lesion evolutions of 3, 4, 8, 12 and 16 weeks. We cannot determine definitively from our results whether sampling the subjects during the acute phase of infection is a cause for obtaining negative ELISA results for some of these case-patients. However, we believe the true value of the information obtained from these ELISA tests would be supportive in a clinical setting. Additionally, we believe this method would be a useful tool in epidemiological studies and in settings where there exists a need to document parasite exposure, as is the case of individuals from non-endemic areas going to work or reside temporarily in endemic areas where they will be subject to parasite contact.

Previous attempts to establish serologic tests as diagnostic or epidemiological tool for leishmaniasis have generated variable results. The reported sensitivity for CL ranging from 23% to 90% success depends on the type, source and purity of the antigen employed (Kar, 1995). To some extent, the cross-reactivity with other microorganisms, particularly Trypanosoma, Plasmodia, Schistosoma and Mycobacterium due to shared epitopes with Leishmania explain the low specificity rates obtained through these methods. Thus, the high sensitivity achieved in our ELISA test may be the result of the novel preparation that allows the capture of native antigens without the contaminating or competing proteins found throughout earlier attempts. Anthony et al. (1980) stated that their failure to develop a sensitive ELISA for cutaneous leishmaniasis was probably a consequence of their inability to eliminate contaminating cell culture proteins. These proteins compete for available sites on the polystyrene surface, thus prohibiting adequate coating with leishmanial antigens resulting in false negative results.

The specificity of these tests have not been thoroughly assessed in the context of this study. Only small groups of endemic normals ($n=20$), malaria ($n=15$) and Chagas ($n=15$) case-patients were evaluated for cross-reactivity to these antigens. In addition, negative results were obtained when 20 Chagas case-patient sera samples from a region in Guatemala known to be endemic for Chagas disease and negative for leishmaniasis indicates the high specificity of the antigen preparations (Ryan, unpublished data). That, coupled with the dramatically different clinical presentations of these diseases from ACL further promotes the use of these ELISA's as a clinical surrogate test and epidemiological tool.

We feel that the improved sensitivity demonstrated here is due to the new antigen preparation techniques involving the use of XOM (Ryan et al., 2002). The immunodominant components of this antigen preparation are under investigation, but initial studies point to the release of native metabolic products, such as nucleoside hydrolases, lipophosphoglycans and glycoproteins that are normally presented to the host upon infection (Cui et al., 2001). In summary, results presented here demonstrate that these ELISAs may become an important tool in the diagnosis and in epidemiological studies of Leishmaniasis.

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