

Variables associated with the post-treatment healing of lesions in patients with American cutaneous leishmaniasis in Paraná State, Brazil

Maria Teresinha Gomes Casavechia¹, Thaís Gomes Verzignassi Silveira¹, Ueslei Teodoro², Vanderly Janeiro¹, Margareth Udo³, Maria Valdrinez Campana Lonardoni^{2*}

Health Sciences Center, Maringá State University, ²Department of Clinical Analysis, Health Sciences Center, Maringá State University, ³Department of Statistics, Exact Sciences Center, Maringá State University.

The purpose of this study was to investigate the relationship of several variables to the healing of lesions in patients with American cutaneous leishmaniasis (ACL). The patients with clinical and/or laboratorial diagnoses of the disease were followed up for varying periods after treatment by clinical evaluation and indirect immunofluorescence assay (IFA), from September 2000 to December 2003. The lesions of 85.3% of the 163 patients had healed by their last return for clinical evaluation, and of these, 82.7% had negative IFA results, indicating an association between the healing of lesions and IFA negativity ($p=0.000$). In patients evaluated up to 120 days after treatment, there was a significant association between negative IFA results and the healing of lesions ($p=0.0000$). Logistic regression analysis showed that negative IFA results on patients' first return after treatment predicted a 2.175 fold greater chance of lesion healing ($p=0.0001$). These results indicate an association between IFA negativity at the first return up to a period of 120 days, and the healing of lesions, and that the chances of healing are significantly higher in patients with negative IFA results at their first return after treatment.

Uniterms: Cutaneous leishmaniasis/treatment. *Leishmania* (*Viannia*) *braziliensis*. Cutaneous lesion/healing.

O objetivo deste estudo foi investigar a associação de algumas variáveis para a cicatrização de lesões em pacientes com leishmaniose tegumentar americana (LTA). Os pacientes com diagnóstico clínico e laboratorial foram acompanhados depois do tratamento por avaliação clínica e reação de imunofluorescência indireta (IFI), de setembro de 2000 a dezembro de 2003. Dos 163 pacientes 85,3% apresentaram cicatrização das lesões no último retorno para a avaliação clínica e 82,7% destes tiveram a IFI negativa indicando uma associação entre a cicatrização das lesões e a negatificação da IFI ($p=0,000$). Nos pacientes acompanhados até 120 dias depois do tratamento houve associação significativa entre os resultados negativos da IFI e a cicatrização das lesões ($p=0,0000$). A análise pela regressão logística mostrou que quando a IFI do primeiro retorno após o tratamento foi negativa, o paciente tinha 2,175 mais chance de cicatrização ($p=0,0001$). Os resultados mostram associação entre a negatificação da IFI e a cicatrização das lesões quando o primeiro retorno foi até 120 dias e que as chances de cicatrização são significativamente maiores nos pacientes que apresentaram IFI negativa no primeiro retorno depois do tratamento.

Unitermos: Leishmaniose cutânea/tratamento. *Leishmania* (*Viannia*) *braziliensis*. Lesão cutânea/cicatrização.

INTRODUCTION

American cutaneous leishmaniasis (ACL) is an ende-

mic disease in Brazil, occurring in every state in the country. It affects people of both sexes and all age groups. The main species of sand flies that cause ACL in Brazil are *Leishmania* (*Viannia*) *braziliensis* (Vianna, 1911), *Leishmania* (*Viannia*) *guyanensis* (Floch, 1954) and *Leishmania* (*Leishmania*) *amazonensis* (Lainson, Shaw, 1972) (Brasil, 2000).

*Correspondence: M. V. C. Lonardoni. Department of Clinical Analysis, Health Sciences Center, Maringá State University, Avenida Colombo 5790, 87020-900 – Maringá - PR, Brazil. E-mail: mvclonardoni@uem.br

ACL has been recorded in the majority of cities in the state of Paraná (Roberto *et al.*, 1997; Silveira *et al.*, 1999; Lima *et al.*, 2002), which accounts for 98% of cases in the southern region of Brazil (Brasil, 2000). The success of ACL treatment is limited. The clinical control of treatment is imprecise, and relapses, even after complete treatment and healing of the initial lesions, have been documented (Guevara *et al.*, 1993). The first-choice drug in Brazil for treating ACL is *N*-methyl glucamine antimoniate (Glucantime®). According to the World Health Organization (WHO, 1995), more than 90% of relapses occur within one year of treatment. Furthermore, the mucosal form may occur among these patients, generally appearing months or even years after the resolution of the skin lesions. Antibodies detected by indirect immunofluorescence assay (IFA) are negative after successful treatment of the disease (Furtado, 1980; Souza *et al.*, 1982). The presence of anti-*Leishmania* antibodies after treatment may signify the persistence of the parasite, and the reduction of antibody titers has been suggested as a tool for the control of treatment efficacy (Chiari, Mayrink, Magalhães, 1973; Mendonça *et al.*, 1988). Therefore, the following-up of patients by clinical and serological evaluation is fundamental (Roberto *et al.*, 1997).

The objective of this research was to study the possible association of the variables sex, IFA at initial diagnosis, IFA at first return, IFA at different post-treatment periods and IFA at last return for clinical evaluation for the healing of lesions in ACL patients. To this end, a group of ACL patients were followed-up for varying periods after clinical and laboratorial diagnosis, after which the contribution of each variable was analyzed.

MATERIAL AND METHODS

Patients

One hundred and sixty-three ACL patients, registered in the 13th Health Region of Cianorte, in northwest Paraná from September, 2000 to December, 2003 were selected. Treatment was prescribed for all patients after positive clinical and/or laboratorial diagnoses. All patients were instructed to return for clinical evaluation and collection of blood samples for the IFA. The follow-up period of the patients varied from 22 to 696 days from the start of treatment, with all patients being evaluated at least once after treatment. The total number of evaluations (including initial diagnosis and subsequent returns) varied from two to five for each patient.

Laboratorial diagnosis

The laboratorial tests for ACL used in this study were Direct Parasite Search (DPS), Montenegro Skin Test (MST), and Indirect Immunofluorescence Assay (IFA). In the DPS, lesion samples were collected by scraping. Smears were made on glass slides, stained by Giemsa, and examined by microscope for the presence of amastigote forms. The MST reagent consisted of *Leishmania (L.) amazonensis* promastigotes containing 40 µg/mL of protein nitrogen, kindly supplied by the Immunobiological Production and Research Center of the Paraná State Health Office. The test was considered positive when an induration diameter greater than or equal to 5mm was observed 48 hours after inoculation. The IFA for IgG was carried out using promastigote forms of *Leishmania (V.) braziliensis*. The serum was diluted, beginning at 1/20. Samples with titers equal to or greater than 40 were considered positive (Silveira *et al.*, 1999).

Treatment protocol

The patients were intravenously treated with *N*-methyl glucamine antimoniate at 15 mg Sb⁵⁺/kg per day (Brasil, 2000). Five patients did not respond to this treatment protocol. Therefore, three of these non-responders were also treated with Pentamidine® (pentamidine isothiocyanate), one with Pentamidine and intralesional infiltration with pentavalent antimonial, and one with intralesional infiltration only. The patients were considered clinically cured after complete healing of their lesions.

Ethical issues

This study complied with Resolution No. 196/96 of the National Health Council of the Brazilian Ministry of Health, and was approved by the Permanent Committee for Ethics in Research Involving Humans of the State University of Maringá (No. 006/2004).

Statistical analysis

An exploratory analysis of the data was carried out and Fisher's exact test was used to verify the associations. Logistic regression was used to verify which variables contributed significantly to the healing of the lesions, using the patients' first return for clinical and serological evaluation as a parameter. The statistical analyses were carried out with the software SAS 8.02 (Statistical Analyses System) using the logistic procedure PROC LOGISTIC with the "stepwise" selection to determine which variables

contributed to healing. Variables were considered significant at the 5% significance level.

The following variables were considered in the logistic regression model: sex, age, IFA at initial diagnosis (IFA_ini), IFA at first return (IFA_ret) and the number of days until the first return (days). The dependent variable was lesion healing by first return (Cret). The following categorizations were used: sex: 1 for male and -1 for female; IFA_ini: 1 if negative and -1 if positive; IFA_ret: 1 if negative and -1 if positive; days: 1 if the first return occurred within a maximum of 120 days and -1 if the first return occurred after 120 days; Cret: 1 if healed and 0 if not healed.

The results found through the statistical analysis showed that the age variable was not significant for the model ($p=0.7507$). Therefore, the adjusted model was given as:

$$P(\text{Cret}=1) = \frac{\exp(\beta_0 + \beta_1 \text{sex} + \beta_2 \text{IFA_ini} + \beta_3 \text{IFA_ret} + \beta_4 \text{days})}{1 + \exp(\beta_0 + \beta_1 \text{sex} + \beta_2 \text{IFA_ini} + \beta_3 \text{IFA_ret} + \beta_4 \text{days})}$$

where: $\beta_0 = 1.6002$, $\beta_1 = -0.5272$, $\beta_2 = 0.6203$, $\beta_3 = 0.7769$ and $\beta_4 = 0.2843$.

RESULTS

Of the 163 cases of ACL studied, 127 (77.9%) were male and 36 (22.1%) female. Patient age ranged between 2 and 79 years (median = 33 years), 8.6% were aged up to 15 years and 6.8% were over 65. All the patients had cutaneous forms of ACL. One hundred and ten patients (67.5%) had a single lesion, 31 (19.0%) had two lesions and 12 (7.4%) had three or more. It was not possible to obtain this information for 10 patients. Two patients had negative DPS results, with IFA and MST not being carried out, and were included in the sample due to their clinical diagnoses.

Table 1 shows that the laboratorial diagnoses of 161 (98.8%) patients were positive for ACL on at least one of the methods used. The DPS was positive in 101 of the 160 patients submitted to the examination (63.1%), while the MST was positive in 134 out of 144 patients (93.1%), and the IFA positive in 141 out of 163 patients (86.5%). The diagnosis was confirmed by all three laboratorial methods in 75 (46.0%) patients. Of the 62 patients with negative results on the DPS, or who were not submitted to the DPS, 40 (64.5%) had positive IFA and MST results, 12 (19.4%) had positive MST results only and 8 (12.9%) had positive IFA results only.

In the follow-up of the 163 patients, 139 (85.3%) showed the healing of lesions by their last return for clinical evaluation (Table II). In 115 (82.7%) of these patients,

IFA was negative, indicating an association between the healing of lesions and the negativity of IFA ($p=0.000$). However, 24 patients (17.3%) continued to have positive IFA results despite having healed lesions. Of the 24 patients that did not show healing of lesions, 17 (70.8%) continued to have positive IFA results.

TABLE I - Results of direct parasite search (DPS), Montenegro skin test (MST) and indirect immunofluorescence assay (IFA), carried out in the diagnosis of American cutaneous leishmaniasis (ACL) in the 163 patients studied from 2000 to 2003

DPS	MST	IFA		Total
		Positive	Negative	
Positive (n=101)	Positive	75	7	82
	Negative	8	0	8
	NP	10	1	11
Negative (n=59)	Positive	37	12	49
	Negative	2	0	2
	NP	6	2	8
NP (n=3)	Positive	3	0	3
	Negative	0	0	0
	NP	0	0	0
Total		141	22	163

NP=Not Performed

TABLE II - Results on indirect immunofluorescence assay (IFA) and of lesion healing in the 163 patients with American cutaneous leishmaniasis (ACL) at last return for clinical evaluation during post-treatment follow-up from 2000 to 2003

Healed	IFA		Total
	Negative	Positive	
Yes	115	24	139
No	7	17	24
Total	122	41	163

Fisher's exact test, $p=0.0000$

Table III shows the results of the clinical and serologic evaluations of the followed-up patients. Of the 81 evaluations performed within 120 days of treatment, 51 patients (63.0%) showed healing of lesions, 41 of whom (80.2%) had negative IFA results. Thirty patients (37.0%) showed no healing of lesions, six of whom (20.0%) had negative IFA results. A significant association ($p=0.0000$) was observed between IFA negativity and the healing of lesions within this evaluation period. Of the 78 patients followed-up from 121 to 210 days, 57 (73.1%) showed

healing of lesions while 42 (73.7%) had a negative IFA. Twenty-one (26.9%) patients from this group showed no healing of lesions, nine of whom (42.9%) had a negative IFA ($p=0.0552$). Of the 51 patients followed-up from 211

TABLE III - Results on indirect immunofluorescence assay (IFA) and for lesion healing in the 163 patients with American cutaneous leishmaniasis (ACL) during different post-treatment periods from 2000 to 2003

Time (days)	Healed	IFA		Total	p
		Negative	Positive		
≤ 120	Yes	41	10	51	0.0000
	No	6	24	30	
Sub Total		47	34	81	
121 to 210	Yes	42	15	57	0.0552
	No	9	12	21	
Sub Total		51	27	78	
211 to 365	Yes	30	11	41	0.0552
	No	4	6	10	
Sub Total		34	17	51	
> 365	Yes	5	5	10	0.3182
	No	0	2	2	
Sub Total		5	7	12	
Total		137	85	222*	

*After initial diagnosis, 114 patients returned once, 42 twice, 5 three times, 1 four times and 1 five times, amounting to a total of 222 blood samples subjected to IFA; p: value of Fisher's exact test: IFA versus healing.

TABLE IV - Logistic regression analysis of factors associated with healing of lesions in 163 patients with American cutaneous leishmaniasis (ACL), followed-up from 2000 to 2003

Variables	Healed		Odds Ratio (95% confidence interval)	Chi-squared	p
	Yes	No			
Gender					
Male	81	46	0.590	3.6662	0.0555
Female	31	5	(0.344 – 1.013)		
IFA on initial diagnosis					
Positive	92	49	1.859	2.3993	0.1214
Negative	20	2	(0.848 – 4.076)		
IFA on First Return\$(within 120 days)					
Positive	30	35	2.175	16.2201	0.0001
Negative	82	16	(1.490 – 3.174)		
Time of First Return					
Within 120 days	49	30	1.329	2.2320	0.1352
>120 days	63	21	(0.915 – 1.930)		

to 365 days, 41 (80.4%) showed healing of lesions, and of these 30 (73.2%) had a negative IFA. Ten (19.6%) of these patients showed no healing of lesions and four (40.0%) had a negative IFA ($p=0.0552$). Finally, of the twelve patients followed-up after 365 days, ten (83.3%) showed healing of lesions, five of whom (50.0%) had a negative IFA. Two (20.0%) patients from this group presented no healing of lesions and showed IFA positivity ($p=0.3182$).

The results of clinical and serologic evaluations performed during other periods (121 to 210 days, 211 to 365 days, and over 365 days) were also analyzed. However, no significant associations between IFA negativity and the healing of lesions were observed for these periods ($p>0.05$).

As a significant association ($p=0.0000$) was observed between IFA negativity and the healing of lesions in the period up to 120 days, logistic regression was performed on the data from this period to analyze the possible factors associated to lesion healing. It was observed that patients who had a negative IFA on their first return after treatment had a 2.175 greater likelihood of their lesions healing than when IFA was positive ($p=0.0001$; CI=1.490-3.174) (Table IV). Analysis of other factors such as gender, IFA result at initial diagnosis, and time of first return after treatment (up to 120 days or after 120 days) found no associations with lesion healing ($p>0.05$).

DISCUSSION

The majority of the patients studied were from the male working-class population, suggesting an extra-domi-

ciliary transmission of ACL, as was previously observed in the north of the state of Paraná (Silveira *et al.*, 1999; Castro *et al.*, 2002). However, the occurrence of cases in the age ranges of up to 15 and over 65 years, for both sexes, also suggests intra- or peri-domiciliary transmission occurred, corroborating data from the north of Paraná State (Roberto *et al.*, 1997; Lima *et al.*, 2002) and on an endemic area of the State of Minas Gerais, Brazil (Passos *et al.*, 2001).

In the present study, IFA showed a higher rate of positivity than the MST or DPS in the diagnosis of ACL, contrasting with results obtained by Silveira *et al.* (1999), who found higher positivity for the MST in patients from the same geographical area. The sensitivity of IFA in the present study was similar to that found in studies on other endemic areas (Marzochi *et al.*, 1980; Lugo de Yarbuh *et al.*, 1996). Together, the three methods used for ACL diagnosis were able to detect 98.8% of the ACL cases, showing it to be a useful combination for diagnosing the disease (Gontijo, Carvalho, 2003).

A highly significant association between the healing of lesions and negative IFA results was observed in this study, confirming that successful treatment reduces levels of anti-*Leishmania* antibodies (Amato *et al.*, 1998). Romero *et al.* (2005) also observed a reduction in antibody levels on enzyme-linked immunosorbent assay (ELISA) post treatment. Therefore, the reduction of anti-*Leishmania* antibody levels after treatment could be an important tool in the control of ACL treatment (Mendonça *et al.*, 1988).

The data for different post-treatment periods was analyzed in order to identify the optimal time after treatment to evaluate the association between the healing of lesions and IFA seroconversion. The analysis showed that lesion healing was associated to negative IFA results when the evaluation was carried out in the period of up to 120 days after treatment. According to Passos *et al.* (2001), the treatment and elimination of the parasites induces lower antigenic stimulation, which contributes to IFA negativity, and Saravia *et al.* (1989) showed that there was an association between longer lesion evolution time and higher IFA titers in patients infected with *L. braziliensis*.

In the present study, a tendency of IFA negativity seen among those patients whose lesions had healed and who were analyzed during the periods of 121 to 210 and 211 to 365 days after treatment ($p=0.0552$). However, no association was observed between IFA negativity and the healing of lesions in those patients evaluated 365 days or more after treatment. This suggests that patients who continue to have antibody titers show a tendency to evolve to the chronic disease, or the reappearance of cutaneous and/or mucosal lesions.

Attention is drawn to the group of patients (17.3%) whose lesions healed but for whom IFA results remained positive. The persistence of antibodies may be an important indicator of the persistence of the parasite, despite the healing of their lesions. Indeed the persistence of the parasite in healed lesions was demonstrated by Mendonça *et al.* (2004). These authors obtained 30 positive PCR and 7 positive IFA results from 32 clinically cured patients, indicating the tendency of *Leishmania* to establish non-apparent infections (Ramirez, Guevara, 1997). Chiari *et al.* (1973) found that 38% of patients submitted to treatment, and considered clinically cured, continued to have positive IFA results. Passos *et al.* (2001) did not associate the risk of recurrence to antibody levels measured by IFA; however, Amato *et al.* (1998) argued that the negativity of IFA antibody titers indicates a lower possibility of recurrence. These observations suggest that a more rigorous following-up of ACL patients, as well as their medicinal dosage and treatment protocol, through clinical and laboratory evaluations, is necessary.

Of the patients whose lesions did not heal, IFA results remained positive for 80.0% and 57.1% during the periods of up to 120 days and from 121 to 210 days, respectively, showing the failure of the treatment. Different degrees of susceptibility to treatment, as well as different levels of immunological response, have been related to the various serodemes (Shaw *et al.*, 1986) of *Leishmania* (*Viannia*) *braziliensis* that circulate in the northwest region of Paraná State (Silveira *et al.*, 1999). While some infections by *Leishmania* present complications during treatment, sometimes producing metastasis and recurrence, others evolve to a spontaneous cure, suggesting that the immunological response is capable of controlling the infection (Brito *et al.*, 2001).

In the present study, it was shown that the chances of lesions healing are greater if the IFA performed is negative when the patient first return after treatment. This indicates that both the initial diagnosis and the start of treatment were probably early. Neither IFA positivity at initial diagnosis, nor the time of the first return for evaluation of treatment, were found to be factors associated with the healing of lesions. No differences in lesion healing between men and women were found, despite the findings of Travi *et al.* (2002), who studied hamsters infected with *Leishmania* (*Viannia*) and observed that the immune response was more permissive to infection among males than females. The factors that contribute to the cutaneous disease evolving into one of the later forms are not fully understood, but delay in healing the first lesion and inadequate treatment are both known predictors (Carvalho *et al.*, 1995).

CONCLUSIONS

The findings of this study confirm that: a) there is an association between IFA negativity at the first return of the patient within 120 days of treatment and the healing of lesions, and b) the chances of healing are significantly higher in patients that showed negative IFA results on their first return for clinical evaluation after treatment.

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