

# DETECTION OF SERUM ANTIBODIES AGAINST *LEISHMANIA* 94 kDA ANTIGEN IN VISCERAL AND CUTANEOUS LEISHMANIOSIS DUE TO *LEISHMANIA INFANTUM*

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## Summary :

*Leishmania* promastigotes polypeptides are analyzed by immunoblotting with sera from patients infected with different *Leishmania* species and presenting visceral or cutaneous infections. These sera recognize *Leishmania* polypeptides in several molecular masses. The major findings of this study are as follow. 1) The *Leishmania* 94 kDa antigen, which is specifically recognized by all sera from *L. infantum*-infected patients with visceral infection, is recognized by some sera from *L. infantum*-infected patients presenting cutaneous infection. 2) All patients with cutaneous infections due to *L. tropica*, *L. amazonensis*, or *L. guyanensis* do not develop anti-94 kDa antibodies, whatever the *Leishmania* species used as antigens. 3) Difference in electrophoretic mobilities is seen between the 94 kDa antigen identified by sera from *Leishmania infantum*-infected patients, and the antigen both recognized by the Conavalin A lectin and a rabbit antiserum raised against deglycosylated Promastigote Surface Protease.

**KEY WORDS :** *Leishmania*, leishmaniosis, immunoblot, antibodies, diagnosis.  
**MOTS CLES :** *Leishmania*, leishmaniose, immunoempreinte, anticorps, diagnostic.

**Résumé :** DÉTECTION D'ANTICORPS DIRIGÉS CONTRE UN ANTIGÈNE DE 94 kDA DANS LES SÉRUMS DE PATIENTS ATTEINTS DE LEISHMANIOSE VISCÉRALE OU CUTANÉE DUES À *LEISHMANIA INFANTUM*.

Des polypeptides de promastigotes de *Leishmania* sont analysés par immuno-empreintes avec des sérums de patients infectés par différentes espèces de *Leishmania* et présentant des infections viscérales ou cutanées. Ces sérums reconnaissent des polypeptides de *Leishmania* de plusieurs masses moléculaires. Les principaux résultats de cette étude sont les suivants: 1) L'antigène 94 kDa de *Leishmania*, reconnu spécifiquement par les sérums de patients infectés par *L. infantum* et ayant une infection viscérale, est reconnu par certains sérums de patients infectés par *L. infantum* et présentant une infection cutanée. 2) Les patients infectés par *L. tropica*, *L. amazonensis*, ou *L. guyanensis*, présentant une leishmaniose cutanée, ne développent pas d'anticorps anti-94 kDa. 3) Une différence de mobilité électrophorétique est observée entre la bande de 94 kDa reconnue par les sérums de patients infectés par *L. infantum*, et celle reconnue à la fois par la lectine concavoline-A et un sérum de lapin dirigé contre la forme déglycosylée de la Protéase de Surface des Promastigotes.

## INTRODUCTION

Protozoans of the genus *Leishmania* include species causing a wide variety of diseases in humans. Symptoms range from self-healing cutaneous lesions to diffuse cutaneous and mucosal manifestations, or severe visceral involvement of the spleen, liver, lymph nodes and bone marrow. Visceral leishmaniosis (VL) is classically caused by species of the *Leishmania donovani* (*L. donovani*) complex: *L. chagasi* in Latin America, *L. donovani* and *L. infantum* in Africa, Asia and the Mediterranean region. *L. chagasi* and *L. infantum* infections can present in two distinct clinical forms. The great majority of cases occur as a VL which is

typically characterized by fever, anemia, hepatosplenomegaly, and weight loss. A marked hypergammaglobulinemia (Shaw and Voller, 1964) and absence of detectable cell-mediated immunity are the principal immunological features of the disease (Carvalho *et al.*, 1981; Carvalho *et al.*, 1985; Sacks *et al.*, 1987; Cillari *et al.*, 1991; Meller-Melloul *et al.*, 1991). The infection has a high mortality rate if untreated and is often complicated by secondary infections. The rarer form is a cutaneous leishmaniosis (CL). *L. infantum* may cause either a single nodule, a larger ulcer, or a mucosal lesion (Rioux *et coll.*, 1980; Bellazoug *et coll.*, 1985; Gramiccia *et al.*, 1987; Gramiccia and Gradoni, 1989; Rioux and Lanotte, 1990; Gradoni *et al.*, 1991; reviewed by Gradoni and Gramiccia, 1994). *L. chagasi* may cause CL (Oliveira *et al.*, 1986; Zeledon *et al.*, 1989; Ponce *et al.*, 1991).

Numerous studies have investigated *Leishmania* antigen expression at the level of specific antibody recognition. Antigens with potential diagnostic value have been selected by their specific recognition by VL sera in Western blot assays (Dos Santos *et al.*, 1987; Reed *et al.*, 1987; Evans *et al.*, 1989; Hoerauf *et al.*, 1992; Mary *et al.*, 1992). In previous studies, using the Western blot technique, we have identified a

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*Leishmania* component of 94 kDa which is recognized by sera from patients with Old and New World VL, but not by sera from patients with other protozooses, helminthiases, fungal and bacterial diseases (L. Rolland-Burger *et al.*, 1991; L. Rolland *et al.*, 1992; L. Rolland *et al.*, 1994). The aim of the present study was to detect anti-94 kDa antibodies in high dilution of sera from patients with VL, and in low dilution of sera from patients with CL due to *L. infantum*, *L. tropica*, *L. amazonensis*, or *L. guyanensis*. While 94 kDa antigen has been found in many species/strains of *Leishmania* (L. Rolland-Burger *et al.*, 1991), a comparison with the Promastigote Surface Protease (PSP) was undertaken.

## MATERIALS AND METHODS

### PARASITES AND CULTURE CONDITIONS

The strains used in this study were : *L. infantum* LEM497 (MCAN/GR/82/LEM497), *L. guyanensis* P30 (IUMB/GF/82/CAYP30), *L. amazonensis* P95 (CAYP95), *L. tropica* LEM135 (MHOM/IQ/LV 556), *L. major* NIH173 (MHOM/IR/83/NIH173), *L. mexicana* LV4 (MNYC/BZ/62/M379), and *L. braziliensis* (MHOM/BR/75/M2904).

Promastigotes were grown at 28°C in culture medium consisting of RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 25 mM sodium carbonate, 4 mM tricine, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

*L. infantum* LEM497 and *L. braziliensis* M2904 strains were maintained by weekly passage *in vitro*. All other strains were maintained by successive *in vivo* inoculations into Balb/c mice; parasites were harvested from cutaneous lesions, and transformation into promastigotes was performed at 28°C in complete culture medium.

### HUMAN SERA

Sera were collected from four patients with parasitologically confirmed VL. Of these four patients, two were from the region of Athens, Greece, one from Chad, and one from the state of Pernambuco, Brazil. CL sera were obtained from three Algerian patients infected with *L. infantum* : all patients were positive for *Leishmania* in biopsy examination, but were negative for classical anti-*Leishmania* antibodies assays. Other CL sera were obtained from 3 Greek patients, and 11 from French Guyana : a brief summary of clinical history and parasitological data for each patient with OW-CL and NW-CL is presented in Table 1. Negative serum was obtained from five healthy French adults with no known past history of leishmaniasis.

### PROTEIN DETERMINATION

Protein concentration was determined using the Pierce protein assay (Pierce Chemical Co., Ill., USA), with bovine serum albumin (fraction V, Sigma Chemical Co., Mo.) as a relative protein standard.

### SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS AND TRANSFER

Between  $1.5 \times 10^8$  and  $2.5 \times 10^8$  promastigotes, corresponding to 1.2 mg protein, were lysed in sample buffer, boiled for five minutes, and subjected to electrophoresis on 10% polyacrylamide gels, according to Laemmli, 1970. Standard markers were a mixture of high- and low-molecular-mass proteins (220 kDa to 14.4 kDa; Pharmacia, Uppsala, Sweden). Electrophoretic profiles of *Leishmania* components were electrotransferred onto nitrocellulose membranes (0.45 mm pore size, Schleicher & Schuell, Dassel, Germany) (Towbin *et al.*, 1979). The markers transferred were stained with India Ink (Hancock and Tsang, 1983).

### WESTERN BLOTTING

Western blotting was performed as previously described (L. Rolland-Burger *et al.*, 1991). Briefly, strips were coated in 100 mM PBS, 5% skimmed milk, and 0.3% Tween 20. Sera were diluted 1:200 (unless stated otherwise) in PBS, 0.1% Tween 20 (washing buffer), and incubated 2 h at 37°C (unless stated otherwise). After washing, strips were incubated with anti-Ig (Nordic, Thilburg, The Netherlands) or anti-IgG (Biosys, Compiègne, France) horse radish peroxidase (HRP) or alkaline phosphatase (AP) (Biosys) diluted conjugates. Enzymatic activities were revealed using  $H_2O_2$  and 3-3', 4-4' diaminobenzidine for HRP conjugates, and nitroblue tetrazolium and bromochloro-indolyl phosphate for AP conjugate.

### ELUTION OF POLYPEPTIDES FROM POLYACRYLAMIDE GELS

Slab gels were cut horizontally between 62 kDa and 98 kDa, by following prestained molecular weight standards run in parallel (14.4 to 200 kDa; Gibco-Bethesda Research Laboratories, Gaithersburg, USA). The selected bands were placed in tubes containing 4% polyacrylamide gels, and were electroeluted overnight at 50 mA. The leads were reversed and the current (50 mA) was again applied for 120 seconds. The samples were dialyzed against double distilled water, overnight at 4°C. Aliquots of 50 µg protein were lyophilized and stored at -80°C until use.

patient				lesion			
n°	sex	age (year)	country (area)	duration	number	size (cm)	ethiological agent
AL1	ND	ND	Algeria	primary infection	1	ND	<i>L. infantum</i>
AL2	ND	ND	Algeria	primary infection	1	ND	<i>L. infantum</i>
AL3	ND	ND	Algeria	primary infection	1	ND	<i>L. infantum</i>
GR1	M	75	Greece (Zakinthos)	relapse	1	ND	ND
GR2	M	45	Greece (Zakinthos)	primary infection	1	3 × 2	<i>L. tropica</i>
GR3	M	88	Greece (Zakinthos)	primary infection	1	ND	ND
FG1	M	41	French Guyana	primary infection	1	ND	<i>L. amazonensis</i>
FG2	M	23	French Guyana	primary infection	6	ND	<i>L. guyanensis</i>
FG3	M	21	French Guyana	primary infection	2	ND	<i>L. guyanensis</i>
FG4	M	30	French Guyana	relapse	2	ND	<i>L. guyanensis</i>
FG5	M	35	French Guyana	relapse	1	ND	<i>L. guyanensis</i>
FG6	M	22	French Guyana	relapse	1	ND	<i>L. guyanensis</i>
FG7	M	24	French Guyana	primary infection	6	ND	<i>L. guyanensis</i>
FG8	M	25	French Guyana	primary infection	1	ND	<i>L. guyanensis</i>
FG9	M	36	French Guyana	primary infection	2	ND	<i>L. guyanensis</i>
FG10	M	21	French Guyana	primary infection	1	ND	<i>L. guyanensis</i>
FG11	M	21	French Guyana	primary infection	2	ND	<i>L. guyanensis</i>

Table 1 – Brief description of clinical history data for the cutaneous leishmaniosis patients analysed in this study. Patients were from Algeria (AL), Greece (GR), and French Guyana (FG). ND: not determined

## LECTIN AFFINOBLOTTING

A two-step procedure was used with Concanavalin A (Con A) according to Faye and Chrispeels, 1985. Strips were saturated for 1 h at 20°C in TBS (20 mM Tris-HCl pH=7.4, 500 mM NaCl) containing 1% Tween 20. Strips were then incubated in washing buffer (TBS containing 0.1% Tween 20, 1 mM CaCl<sub>2</sub>, and 1 mM MnCl<sub>2</sub>) containing 25 mg/ml of Con A (Sigma). After 1 h at 20°C, strips were washed and incubated in washing buffer containing 50 mg/ml HRP (Boehringer Mannheim, Mannheim, Germany). After 1 h at 20°C, the strips were washed and immersed in the staining solution 0.27 ml H<sub>2</sub>O<sub>2</sub> (110V) and 0.5 mg 3,3'-4,4' tetrachlorhydrate diaminobenzidine (Sigma) per ml TBS. Controls were treated according to the same protocol, except for the inclusion of 200 mM methyl-alpha-D-mannoside (Sigma), or 15 mM methyl-alpha-glucoside (Sigma) in the buffers used for washing, Con A binding, and HRP binding.

A one-step procedure was used with the HRP-labeled lectin Ricinus Communis Agglutinin RCA120 (Sigma) (Faye and Salier, 1989). Strips were saturated for 1 h at 20°C in TBS with 0.1 % Tween 20 (washing buffer), then incubated in washing buffer containing 5 µg/ml HRP-labeled RCA120. After 90 min at 20°C, strips were washed and immersed in the staining solution consisting of 0.3 ml H<sub>2</sub>O<sub>2</sub> (110V) in 110 ml TBS mixed with 30 mg 4-chloro-1-naphtol (BioRad, Richmond, Ca.) in 10 ml methanol.

## LEISHMANIA PROMASTIGOTE SURFACE PROTEASE (PSP) DETECTION

Strips were coated for 1 h at 20°C in 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 5% skimmed milk, and 0.05% sodium azide. Strips were then incubated for 16 h at 20°C with a rabbit antiserum directed against the chemically deglycosylated-gp63 (Bouvier *et al.*, 1985) diluted 1:500 in coating buffer. After 3 washes, strips were incubated 1 h with 0.5 mg/ml HRP-Protein A (Sigma) in coating buffer. After three washes, the peroxidase activity was revealed with 0.01% H<sub>2</sub>O<sub>2</sub> and 0.3% 4-chloro-1-naphtol in methanol : 10 mM TBS (1V:3V). As positive control, 1 mg of PSP was submitted to electrophoresis, blotted onto nitrocellulose, then incubated with the rabbit anti-PSP serum, in the same conditions.

## RESULTS

### VL DUE TO *L. INFANTUM*

Here we report about quantitative aspects of the recognition of the 94 kDa antigen by VL sera. Serum was collected from a 21 year-old Greek patient with classical VL : at this time hepatosplenomegaly was present, and *Leishmania* amastigotes were detected in sternal bone marrow aspirate. This serum was tested for IFAT and had antibody titer of 1600. IgG, IgM, IgA, and IgE reactivity to SDS-PAGE separated *L. infantum* (LEM497)



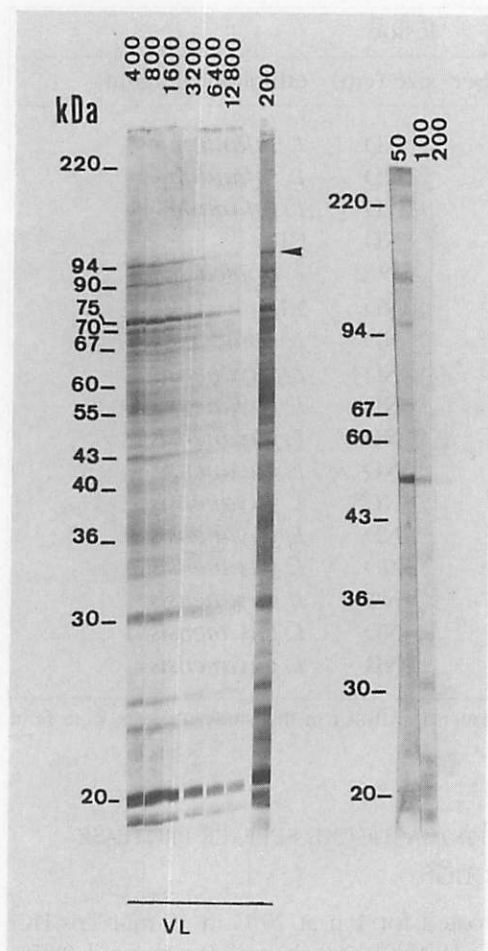


Fig. 1. - Immunoblot of *L. infantum* LEM497 polypeptides reacted with the serum of a Greek patient with VL (left) and with a French negative serum (right). Sera were incubated at dilutions indicated at the top of the blots : from 200 to 12,800 for VL serum, and from 50 to 200 for negative serum. HRP-labeled goat anti-human IgG as second antibody (1:5000). Arrow indicates the 94 kDa antigen position.

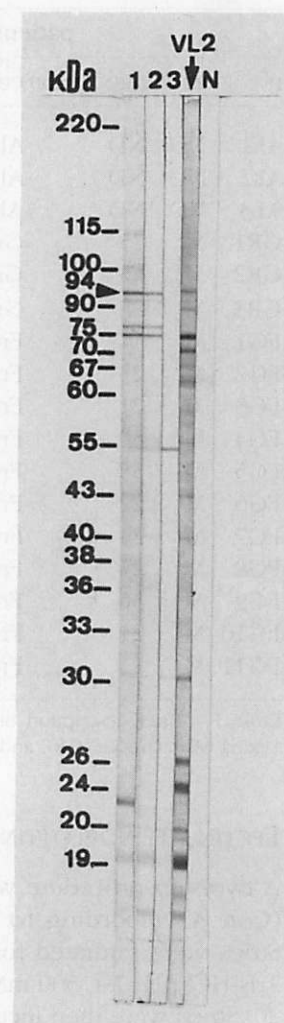


Fig. 2. - Immunoblot of *L. infantum* LEM497 promastigote polypeptides reacted with *L. infantum*-infected patients sera. Lanes : 1-3, individual CL Algerian patient sera (1:50); VL2, individual VL Tchad patient serum (1:200). AP-labeled anti-human IgG as second antibody (1:2500).

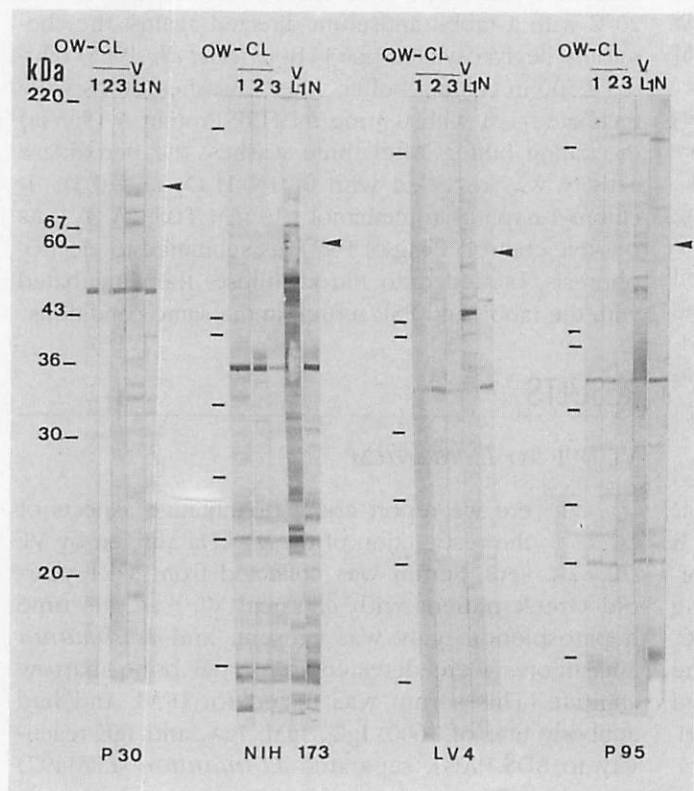


Fig. 3. - Immunoblot of polypeptides from different *Leishmania* species (*L. guyanensis* P30, *L. major* NIH173, *L. mexicana* LV4, *L. amazonensis* P95) reacted with *L. tropica*-infected patient sera. Lanes : 1-3, individual CL Greek patient sera (1:100); VL1, individual VL Greek patient serum (1:200); N, pool of French negative sera (1:100). HRP-labeled anti-human Ig (G, A, M) as second antibody (1:1,000). Arrow indicates the 94 kDa antigen position.

polypeptides blotted onto nitrocellulose were then tested for 2 h at 37°C. No IgA and IgE were detected, some IgM were detected, in particular IgM anti-94 kDa (data not shown). In this serum, IgG anti-94 kDa antibodies were detectable at the 12,800 serum dilution (Fig. 1, left, arrow). In contrast, a 200, 100, or 50 fold diluted French negative serum does not detect the 94 kDa antigen (Fig. 1, right).

#### CL DUE TO *L. INFANTUM*

Sera from three Algerian patients infected with *L. infantum* and presenting CL, were tested for 2 h at 37°C, for IgG reactivity to *L. infantum* LEM497 polypeptides (Fig. 2). The seroreactivity patterns of *L. infantum* CL sera were distinguishable from those of *L. infantum* VL sera by the fewer immunostaining of *L. infantum* polypeptides. Indeed, in contrast to the antigen-binding pattern observed with the serum of a VL patient from Chad (lane VL2), the sera from three Algerian CL patients primarily identified antigens of 94, 92, 77, 75, 23 and 19 kDa (lanes 1-3). Of particular interest, two out of the three CL sera identified the 94 kDa antigen (lanes 1, 2, arrow).

#### CL DUE TO *L. TROPICA*, *L. AMAZONENSIS*, OR *L. GUYANENSIS*

Sera from three Greek patients with OW-CL were tested for Ig reactivity to OW (*L. major* NIH173), as well as NW (*L. guyanensis* P30, *L. mexicana* LV4, *L. amazonensis* P95) *Leishmania* polypeptides (Fig. 3, lanes 1-3). Sera identified few or no antigens, whatever the *Leishmania* species used as antigens for the immunoblot.

Similarly, sera from 11 French Guyana patients with NW-CL were tested for Ig reactivity to OW (*L. tropica* LEM135), and NW (*L. guyanensis* P30, *L. mexicana* LV4, *L. amazonensis* P95) *Leishmania* polypeptides (Fig. 4, lanes 1-11). Responses are slightly different, depending of the *Leishmania* species used for the immunoblotting. These 11 NW-CL patient sera primarily identified antigens of 55 kDa to 96 kDa with *L. guyanensis* P30 blot, and antigens of 55 kDa to 70 kDa with *L. tropica* LEM135, *L. mexicana* LV4, and *L. amazonensis* P95 blots.

Neither *L. tropica*-infected nor *L. guyanensis*-infected patients sera could recognize the 94 kDa antigen identified by the VL1 Greek patient serum (Fig. 3 and fig. 4, lanes VL1, arrows).

#### 94 kDa ANTIGEN AND PSP DETECTIONS

Electroeluted *L. braziliensis* (M2904) fraction (62 kDa < Mr < 98 kDa) was re-electrophoresed, then transferred onto nitrocellulose membrane. India ink staining

revealed 9 bands (28 kDa, 40 kDa, 63 kDa, 67 kDa, 70 kDa, 90 kDa, 94 kDa, 96 kDa, and 98 kDa (Fig. 5 : lane 1). Most of them are recognized by the Brazilian VL serum (lane 2) and by Con A, with a major band at 70 kDa, that reveal the presence of  $\alpha$ -D-mannose and/or glucose terminal residues (lane 3). Binding with Con A was completely blocked by incubation with 200 mM methyl- $\alpha$ -D-mannoside (lane 5), but not with 15 mM methyl- $\alpha$ -glucoside (lane 4). HRP-labeled RCA120 did not bind to the *L. braziliensis* fraction (lane 6).

The reactions of anti-*L. major*-PSP rabbit antiserum on *L. braziliensis* fraction and on purified *L. major*-PSP were compared. The purified *L. major*-PSP was strongly stained by the rabbit antiserum (lane PSP), whereas the *L. braziliensis* fraction was very slightly stained by the antiserum (lane 7, arrow b). The PSP band comigrates with the major Con A band (compare lane 7 to 3, arrow b). As can be seen, relative molecular mass of the 94 kDa (arrow a) and of PSP (arrow b, around 70 kDa) are different.

## DISCUSSION

Two forms of disease can develop in patients infected with *L. infantum*, a limited form of cutaneous leishmaniasis, or a fatal systemic form termed VL or Kala-azar, in which parasites develop in viscera. Differences in antigen expression by natural variants of *L. infantum* may induce the development of lymphocytes with different effector functions in patients with the two forms of disease. In the present study, we have analysed *L. infantum* LEM497 promastigotes (a viscerotropic strain) by immunoblotting with sera from *L. infantum*-infected patients with VL or CL. By comparing some VL and CL patient antibody responses against antigens expressed by *L. infantum* LEM497 parasites, we found that the antigen-binding patterns were different. Blot analyses of *Leishmania* lysates with VL sera showed that they react with the majority of the polypeptides present in the total *L. infantum* extract. This confirms that high levels of *Leishmania*-specific antibodies are produced during VL. In contrast, few *L. infantum* polypeptides are identified by sera from *L. infantum* infected-patients presenting cutaneous lesion. One possible explanation for this finding is related to differences in the 'parasite load' of the VL and CL patients. The VL patients contain more parasites than do CL patients. Moreover, the distribution of parasites in VL patients viscera may induce different recruited B-cell clones than the parasites found in the lesions of patients with CL.

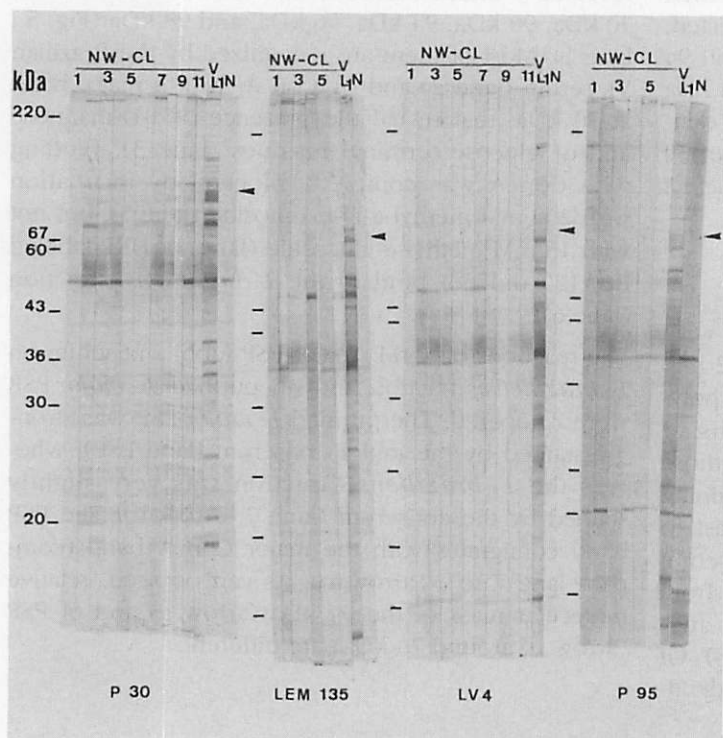


Fig. 4. – Immunoblot of polypeptides from different *Leishmania* promastigotes species (*L. guyanensis* P30, *L. tropica* LEM135, *L. mexicana* LV4, *L. amazonensis* P95) reacted with *L. amazonensis*- or *L. guyanensis*-infected patient sera. Lanes : 1-11, individual CL sera from French Guyana patients (1:100); VL1, individual VL Greek patient serum (1:200); N, pool of French negative sera (1:100). HRP-labeled anti-Ig (G, A, M) as second antibody (1:1,000). Arrow indicates the 94 kDa antigen position.

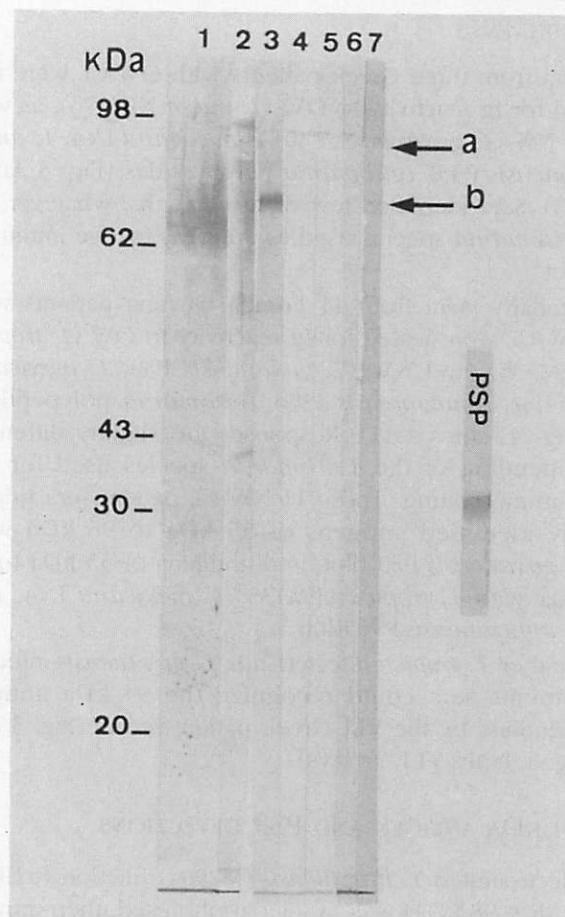


Fig. 5. – Differential affinity of Con A, RCA120, and serum anti-PSP for a *L. braziliensis* (M2904) fraction (98 kDa > Mr > 62 kDa) blotted onto nitrocellulose membrane. Sheets were treated with: lanes: 1, India ink; 2, individual Brazilian VL serum (1:100); 3, Con A + HRP; 4, Con A + HRP in the presence of 15 mM methyl- $\alpha$ -glucoside; 5, Con A + HRP in the presence of 200 mM methyl- $\alpha$ -D-mannoside; 6, HRP-labeled RCA120; 7, rabbit antibodies raised against deglycosylated *L. major* PSP. Arrow (a) indicates the 94 kDa antigen position; arrow (b) indicates the 70 kDa position.



The *L. infantum* infected-patient sera tested in this study showed various degrees of heterogeneity in antibody reactivity to *L. infantum* polypeptides. This was observed in both VL and CL patients. We observed that in general, each patient responded to only a subset of antigen expressed by parasites. As suggested by Mengistu *et al.*, 1990, reporting heterogeneity in serum antibody specificities to *L. aethiopica* antigens in patients with localized and diffuse CL, it is possible that these nascent reactivities play an influential role in determining the type of immune response which any one individual will make after encountering these antigens during the infectious process.

Similarly, CL sera from *L. tropica*, *L. amazonensis*, or *L. braziliensis*-infected patients, reacted in a heterogeneous manner with few *Leishmania* polypeptides, whatever the *Leishmania* strain used. The responses of *L. infantum*-infected CL patients were less strong than those of the *L. amazonensis* and *L. braziliensis* CL patients.

In previous studies, in spite of heterogeneity of antibody responses, we have identified a *Leishmania*-94 kDa component which was recognized by Old World (*L. Rolland-Burger et al.*, 1991) as well as New World (*L. Rolland et al.*, 1994) VL patients sera, but not by other infection sera, among them cutaneous leishmaniasis, Chagas' disease, malaria, tuberculosis, and leprosy. The specific detection of these antibodies could be important in the serological diagnoses of VL in areas in which Chagas' disease, tuberculosis, or leprosy are co-endemic, particularly if these antibodies recognizing the 94 kDa antigen are elicited early in the infection. Here, we report that the 94 kDa antigen is recognized by VL sera at high serum dilution. A minimum of 5 min for serum incubation, in a standard procedure (1:200 serum dilution, incubation for 2 h at 37°C), has been necessary to visualize the 94 kDa band (data not shown).

In contrast, two out the three CL sera from *L. infantum*-infected patients identified this antigen. There are striking differences between the pattern of antigens recognized by CL serum 3, compared with the other two. However, there are no known striking differences between clinical and parasitological status of the CL patient 3, compared to the other two. Thus, the recognition of the *Leishmania* 94 kDa antigen is not related to the clinical picture of the *L. infantum*-infected patients. The 94 kDa component may be a common molecule to natural variants of *L. infantum*. Numerous molecules have been characterized in different species of *Leishmania*, in particular surface molecules, including lipophosphoglycan (LPG), PSP, transporters and ectoenzymes (reviewed in Chang *et al.*, 1990; Alexander and Russell, 1992; Schneider *et*

*al.*, 1992). PSP, with molecular mass of 63 kDa, is a membrane metalloprotease expressed at the promastigote surface of all *Leishmania* that have been examined (Bouvier *et al.*, 1985; Etges *et al.*, 1986; Bouvier *et al.*, 1987; Bouvier *et al.*, 1989; Etges *et al.*, 1992). Antigenic and structural relationships between PSP of different *Leishmania* promastigote species have been reported (Bouvier *et al.*, 1985; Etges *et al.*, 1985; Lemesre *et al.*, 1985; Bouvier *et al.*, 1987). Anti-PSP antibodies have been detected in sera from CL and VL patients; PSP is thus potentially useful antigen for immunodiagnosis of leishmaniasis (Reed *et al.*, 1990). The 94 kDa antigen has been identified by VL sera, on different *Leishmania* promastigote species : *L. infantum*, *L. donovani*, *L. tropica*, *L. major*, *L. guyanensis*, *L. mexicana* (Rolland-Burger *et al.*, 1991), *L. amazonensis* (Rolland *et al.*, 1994) and *L. braziliensis* (present study). This suggests the existence of common antigenic epitope(s) between the 94 kDa antigens of different *Leishmania* promastigote species. Interestingly, we showed that the 94 kDa antigen was not identified on *L. infantum* blots by 1:200 diluted sera from 40 CL patients (Greece, Bolivia, French Guyana) (Rolland-Burger *et al.*, 1991), nor by 1:50 diluted sera from 26 CL patients on four *Leishmania* species blots (Iran, Bolivia, Brazil) (Rolland *et al.*, 1994). Similarly, in the present study, no anti-94 kDa antibodies have been identified, on five *Leishmania* species blots, in 1:200 diluted sera from 14 patients with CL due to *L. tropica*, *L. amazonensis*, or *L. guyanensis*. This differential immunogenicity may be related to differences in antigen processing.

We have electroeluted the 62-98 kDa region from SDS-PAGE gels of a *L. braziliensis* promastigote lysate. Two pieces of evidence suggest that the *Leishmania* 94 kDa antigen is not PSP, the major promastigote surface protein of *Leishmania*. Firstly, the 94 kDa antigen (stained with VL sera) migrates above of *L. braziliensis* gp63 (identified by an affinity purified rabbit antibodies directed against chemically deglycosylated *L. major* gp63 (Bouvier *et al.*, 1985). Secondly, HRP-Con-A does not stain the 94 kDa antigen, but stains a band that comigrates with that recognized by the rabbit antibodies.

In conclusion, a *Leishmania*-94 kDa antigen, different from *Leishmania* PSP, was recognized by serum antibodies from *L. infantum*-infected patients. This antigen is not identified by serum antibodies from *L. tropica*-, *L. amazonensis*-, or *L. guyanensis*-infected patients. Thus, differential immunogenicity (circulating anti-94 kDa antibodies in *L. infantum*-infected patients sera but not in other infection patient sera) makes these antibodies immunological markers of the

*L. infantum* infection. The observed sensibility of the detection of such antibodies (presence in all *L. infantum*-infected VL patients but not in all *L. infantum*-infected CL patients) confers to our immunoblot method its value in the serodiagnosis of the visceral form of the disease.

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