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HUMAN CUTANEOUS LEISHMANIASIS CAUSED BY *Leishmania (Viannia) braziliensis* IN SANTIAGO DEL ESTERO, ARGENTINA: IDENTIFICATION OF PARASITES BY MONOCLONAL ANTIBODIES AND ISOENZYMES

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SUMMARY

Diagnostic and parasite characterization and identification studies were carried out in human patients with cutaneous leishmaniasis lesions in Santiago del Estero, Northern Province of Argentina. Diagnostic procedures were biopsies of lesions for smears and inoculations in hamster, needle aspirations of material from ulcers for "*in vitro*" cultures. Immunodiagnostic techniques applied were IFAT-IgG and Montenegro skin test. Primary isolation of eight stocks of leishmanial parasites was achieved from patients with active lesions. All stocks were biologically characterized by their behaviour in hamster, measurements of amastigote and promastigotes and growth "*in vitro*".

Eight stocks were characterized and identified at species level by their reactivity to a cross-panel of sub-genus and specie-specific Monoclonal Antibodies through an Indirect Immunofluorescence technique and a Dot-ELISA.

We conclude from the serodeme analysis of Argentina stocks that: stocks MHOM/AR/92/SE-1; SE-2; SE-4; SE-8; SE-8-I; SE-30; SE-34 and SE-36 are *Leishmania (Viannia) braziliensis*. Three *Leishmania* stocks (SE-1; SE-2 and SE-30) did not react with one highly specie-specific Monoclonal Antibody (Clone: B-18, *Leishmania (Viannia) braziliensis* marker) disclosing two serodeme group patterns.

Five out of eight soluble extracts of leishmanial promastigotes were electrophoresed on thin-layer starch gels and examined for the enzyme MPI, Mannose Phosphate Isomerase; MDH, Malate Dehydrogenase; 6PGD, 6 Phosphogluconate Dehydrogenase; NH, Nucleoside Hydrolase, 2-deoxyinosine as substrate; SOD, Superoxide Dismutase; GPI, Glucose Phosphate Isomerase and ES, Esterase. From the isoenzyme studies we concluded that stocks: MHOM/AR/92/SE-1; SE-2; SE-4; SE-8 and SE-8-I are isoenzymatically *Leishmania (Viannia) braziliensis*. We need to analyze more enzymes before assigning them to a *braziliensis* zymodeme.

KEYWORDS: Cutaneous leishmaniasis; *Leishmania (Viannia) braziliensis*; Identification; Santiago del Estero, Argentina.

INTRODUCTION

The mucocutaneous and visceral leishmaniasis have been known in Argentina territory since the implementation of the Mision de Estudios de Patologia Regional Argentina (MEPRA) directed by Mazza in the Jujuy Province in 1926[13,14]. Soon after, BERNASCONI (1930) published an important report on the presence of leishmania-

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sis in the provinces of Jujuy, Salta and Santiago del Estero. More recently clinical and therapeutic studies have identified the presence of endemic tegumentary leishmaniasis in at least nine Northern argentinian provinces[6,8]. Nonetheless little is known about detailed taxonomic identification of the parasites present in such endemic areas and their geographic distribution. As established by PETERS et al., (1983) it is of fundamental importance in an endemic area the identification of the species of *Leishmania* causing the human leishmaniasis. The better initial treatment and subsequent management of a patient will depend of a complete identification of the parasite.

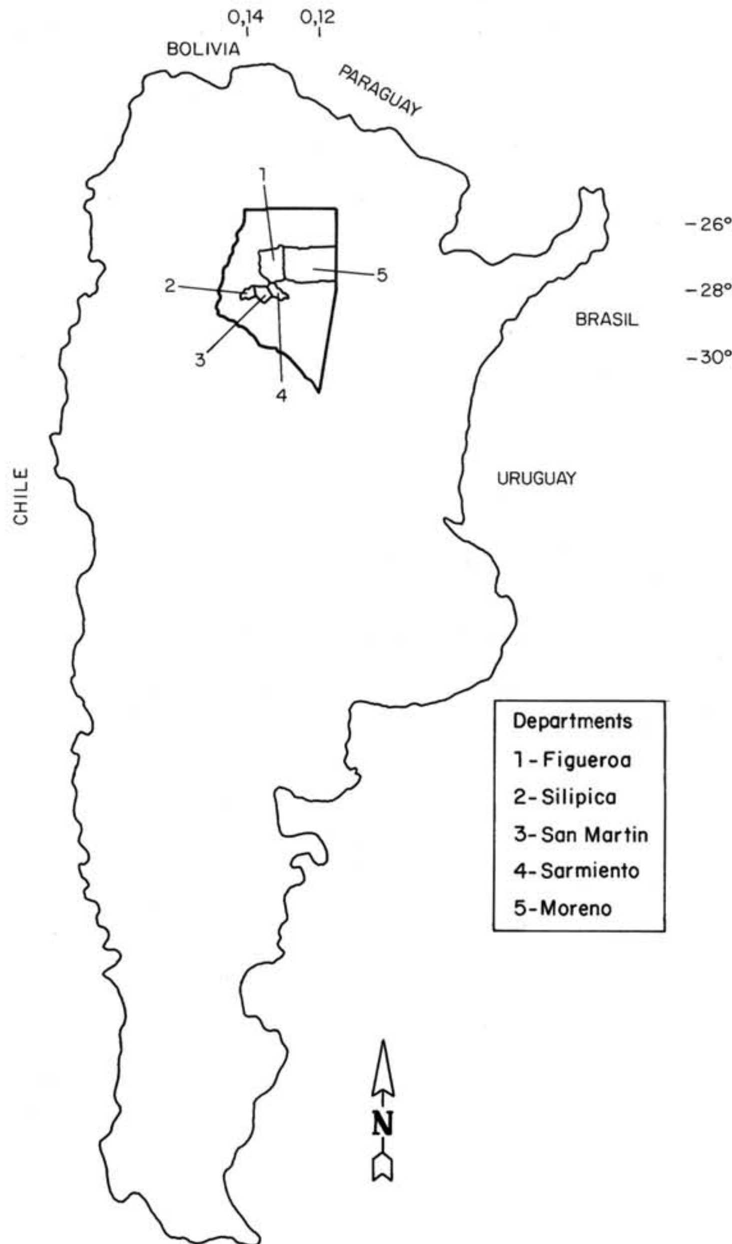

Fig. 1 - Province of Santiago del Estero - Argentina.

The present work describes a parasitological study of *Leishmania* isolates from human patients with cutaneous lesions compatible with the disease. By biological and molecular methods the isolates from the Argentinian province of Santiago del Estero were classified as *Leishmania (Viannia) braziliensis*.

MATERIAL AND METHODS

Parasites and Patients

The Dermatology Unit of the Hospital Independencia, in Santiago del Estero, Province of Argentina, performed the clinical examination of patients with lesions suggestive of leishmaniasis. Patient's data were recorded on a clinical case report form. Information relating to the site, size and nature of the skin lesions were recorded. All diagnosis and isolation of the parasite were done at the same hospital. At laboratories of Universidad Nacional del Sur, Bahia Blanca eight Argentinian leishmanial stocks were studied following the methodology described by CUBA CUBA et al., (1985). All were isolated from skin ulcers of human patients. In most cases the cutaneous lesions were single. Most commonly, the lesions were extensive ulcers with a high indurated erythematous border. Verrucose lesions were also present. Figure 1 include the localities of the Province of Santiago del Estero in Argentina, where all the patients investigated were from.

For parasite demonstration small tissues samples were removed by punch (4 mm). Smears

prepared and stained by Giemsa and searched for amastigotes. Then biopsy samples were homogeneized in tissues grinder and the suspensions inoculated in the dorsal paw of two hamsters (*Mesocricetus auratus*). Material obtained by needle aspiration of the borders of the lesions were cultured in Difco Blood agar and Schneider's *Drosophila* media[3]. The tubes incubated at 23°C were examined every week for two months. Hamsters were observed weekly. If some lesions was detected reisolation of parasites was attempted and necropsies done in all animals for assessment of metastasis and visceralization. Smears from rodent lesions were submitted to morphometric studies of amastigotes as well as the stained promastigotes from cultures. The Argentinian isolates were compared with the World Health Organization reference strains of *Leishmania* from New World (London School of Hygiene and Tropical Medicine, WHO Reference Centre and Brasilia University Cryobank – leishmaniasis Regional Reference Laboratory). All classified Argentinian strains are at present deposited at WHO/Reference Centre Cryobank – London, England.

Measurements on amastigotes and promastigotes were done on 100 organisms per each stock. Standard micrometry were carried out using an calibrated ocular micrometer (Graticules Ltd, Tonbridge Kent, England).

Immunodiagnosis

Immunological diagnosis was based on Montenegro's skin test (MST), done on right forearm, of the patients using a promastigote antigen containing 40 ug Nitrogen protein/ml prepared at Brasilia University (CUBA CUBA et al., 1985). Specific antibodies were tested using an Indirect Immunofluorescence Assay (IFAT-IgG), according to CUBA CUBA et al., 1981.

Isoenzymes Studies

Soluble extracts of five leishmanial stocks were submitted to electrophoresis on thin-layer (1 mm thick) starch gels (GODFREY & KILGOUR, 1976) using the electrophoretic conditions and enzyme visualization methods described by EVANS et al., 1984. The following enzymes were examined: Nucleoside Hydrolase (NH(D), 2-deoxyinosine as substrate- E.C. 3.2.2.2.1); Superoxide Dismutase (SOD, E.C. 1.1.5.1.1.); Glucose Phosphate Isomerase (GPI, E.C. 5.3.1.9.) and Esterase (ES, E.C. 3.1.1.1.) all of which clearly distinguish between the sub-genera *Leishmania (Viannia)* and *Leishmania (Leishmania)*.

6-Phosphogluconate Dehydrogenase (6-PGD, E.C. 1.1.1.44) which distinguishes *L. (Viannia) braziliensis* from *L. (Viannia) guyanensis* but not *L. (Viannia) braziliensis* from *L. (Viannia) peruviana*.

TABLE 1

Clinical and diagnostic features of argentinian patients with cutaneous leishmaniasis lesions from whom parasites were isolated: Santiago del Estero, Argentina, 1992.

Patient code (Departamento)	AGE/SEX	Cutaneous Lesions	Diagnostic Methods					
			Smears	Culture Media		Hamster Inoculation	Skin Test**	Serology (IFAT)
				Difco (BA)	Schneider's			
SE-1 (Silipica)	45/F	SINGLE	+	+	–	–	+	1:160
SE-2 (Silipica)	42/M	SINGLE	+	+	+	+	+	1:320
SE-4 (San Martin)	14/M	SINGLE	–	+	Contaminated	–	+	1:80
SE-8* (Sarmiento)	8/F	MULTIPLE	+	+	+	–	+	1:640
SE-8.1*			+	+	+	+	+	
SE-30 (San Martin)	37/M	MULTIPLE	+	+	–	Not Done	+	1:160
SE-34 (Figueroa)	11/M	SINGLE	+	+	+	+	+	1:160
SE-36 (Moreno)	11/F	SINGLE	+	+	+	+	+	1:80

* Parasites isolated from two different lesions of the same patient

** Montenegro skin test: antigen concentration 40 μg N/ml. Promastigotes *L. (L) amazonensis*.
Dose: 0.1 ml – Control phenol-saline.

Mannose Phosphate Isomerase (MPI, E.C. 5.3.1.8.) and Malate Dehydrogenase (MDH, E.C. 1.1.1.37) enzymes are described as markers that can be used in thin-layer starch gel electrophoresis to differentiate between *L. (V) braziliensis* and *L. (V) peruviana*[1].

The following WHO references strains were used: MHON/BR/83/LTB-300, *L. (V) braziliensis*; MHOM/BR/75/M-4147, *L. (V) guyanensis*; MHOM/PA/71/LS-94, *L. (V) panamensis*; MHOM/PE/84/LC-37, *L. (V) peruviana*; MCAN/PE/92/LRP-737, *L. (V) peruviana*; MHOM/BEL/88/BEL-89, *L. (Viannia)*; INS/BR/77/PH8, *L. (Leishmania) amazonensis*. The test stocks were: MOHM/AR/92/SE-1; SE-2; SE-4; SE-8 and SE-8I.

Monoclonal antibodies: Indirect Immunofluorescence Assay (IFAT) and Direct Immunodot Assay.

The panel of Monoclonal Antibodies (Mabs) used in this study, specific for members of the sub-genera *Leishmania (Viannia)* and *Leishmania (Leishmania)* have been listed in Table 3. The promastigotes were cultured in Schneider's medium (20% fetal bovine serum). Identification of the *Leishmania* was performed with the IFAT using MAbs clones from mice ascitic fluids and the procedure followed the protocol from McMAHON-PRATT et al., 1984.

Preliminary observations using a sligthly modified Immunodot Assay/MAbs by direct binding of promastigotes on membranes (Nylon, Hybond+) and

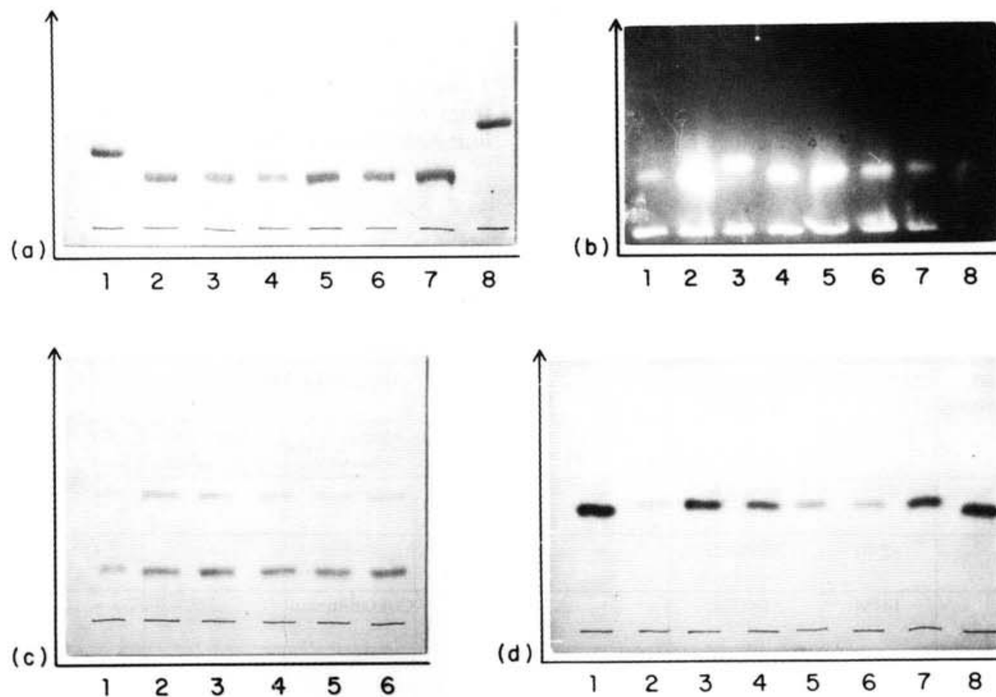

Fig. 2 – Thin layer starch gel electrophoresis of the enzymes (a) 6PGD. (b) ES. (c) NH (d) NH (D).
(a) 6PGD 1. *L. (V) guyanensis (M4147)*. 2. *L. (V) braziliensis (LTB-300)*. 3. *Argentinian stock (SE-1)*. 4. *Argentinian stock (SE-8)*. 5 *Argentinian stock (SE-2)*. 6. *Argentinian stock (SE-8 I)*. 7. *Argentinian stock (SE-4)*. 8. *L. (L) amazonensis (PH8)*.
(b) ES. 1. *L. (V) braziliensis (LTB-300)*. 2. *L. (V) guyanensis (M4147)*. 3. *L. (L) amazonensis*. 4. *Argentinian stock (SE-1)*. 5 *Argentinian stock (SE-8)*. 6. *Argentinian stock (SE-2)*. 7. *Argentinian stock (SE-8 I)*. 8. *Argentinian stock (SE-4)*.
(c) NH 1. *L. (V) braziliensis (LTB-300)*. 2. *Argentinian stock (SE-1)*. 3. *Argentinian stock (SE-8)*. 4. *Argentinian stock (SE-2)*. 5. *Argentinian stock (SE-8 I)*. 6. *L. (V) peruviana (LC-37)*.
(d) NH(D) Lanes 1-5 as in (c), except 6. *L. (V) peruviana (LRP 737)*. 7. *L. (V) peruviana (LC-37)*. 8. *L. (V) guyanensis (M4147)*.

TABLE 2

Occurrence of Metastasis in the hamster (*Mesocricetus auratus*) after intradermal inoculation of amastigotes from human Cutaneous *Leishmania (Viannia) braziliensis* infection.

Tissues Sample	Organs of Hamster Searched (Between 18-45 days):	Demonstration of Leishmania by:	
		Smears	Culture*
SE-1	⇒ Para-axiliary Lymph-node	+	+
SE-1	⇒ Spleen	–	–
SE-1	⇒ Liver	+	+
SE-1	⇒ Bone Marrow	–	–
SE-2	⇒ Para-axiliary Lymph-node	+	+
SE-2	⇒ Spleen	–	–
SE-2	⇒ Liver	–	–
SE-2	⇒ Bone Marrow	+	+

* 3 Tubes of Difco Blood Agar per Organ.

Horseradish Peroxidase immunobloting were performed according to PATEL, et al., 1988. For sample preparation *Leishmania* promastigotes were obtained from cultures in Schneider's medium. Pre-washed forms were counted and adjusted to a final concentration of 10^8 promastigotes/ml. The antigens were prepared by placing 20 ul of parasite suspension on Hybond C nitrocellulose discs (5 mm diameter) and then air dried for 30 min. For immunoenzymatic labelling the samples were incubated in 0.3% H_2O_2 in TBS (Tris Buffered Solution) for 5 min to neutralize the endogenous peroxidase activity. The discs were then rinsed with TBS and immersed in TBS/Tween 20/2% casein blocking solution for 20 min under shaking in rocker in humid chamber at room temperature. After washing (x3) in TBS the discs were incubated for 60 min with the respective monoclonal antibody

TABLE 3

Summary of results of IFAT using monoclonal antibodies: serodeme analysis of *Leishmania* stocks from Santiago del Estero, Argentina, 1992.

Leishmania sp	Monoclonal Antibody (Specificity)*	SE-1	SE-2	SE-4	SE-8	SE-8 I	SE-30	SE-34	SE-36	LTB-300**
1) XIII 3E6-BII (B-16)	L. (V) b	++	++	++	+++	+++	++	++	+	+++
2) XIII 3F4-F6 (B-17)	L. (V) b	+	+	+	++	++	++	+	+	++
3) XIII 2A5-A10 (B-18)	L. (V) b	–	–	+	+++	++	–	+	+	+++
4) VI 4B9-D10 (B-2)	L. (V)	++	+	++	++	++	+	++	++	+++
5) VII 2C5-C5 (B-5)	L. (V)	++	++	+++	+++	+++	++	++	++	+++
6) XLIV 5A2-B9 (B-19)	L. (V) g	–	–	–	–	–	–	–	–	–
7) IX 2H7-E10 (M2)	L. (L) a	–	–	–	–	–	–	–	–	–
8) IX IF9-D8 (M4)	L. (L) a	–	–	–	–	–	–	–	–	–
9) LXVIII 4E12-D8 (M12)	L (L) m	–	–	–	–	–	–	–	–	–

* IFAT = Indirect immunofluorescent assays (twice), working dilutions: 1:100-1:500
MABS = Ascitic fluids
** MHOM/BR/83/LTB-300 (WHO, Reference strain)
+++ = Strong definite positive signal (Flagellar pocket, Membrane, flagellum)
++ = Positive signal (membrane, flagellum)
\+ = Scored positive although low signal (Membrane)
– = Negative

(working dilution 1:500) at room temperature and shaking. Washed (x3) in TBS wash solution and incubated with the Peroxidase Conjugate anti-mouse IgG (goat Jackson Lab UK), 1:1000 working dilution in TBS/Tween 20/2% casein for 60 min at room temperature. After washing (x3) the substrate solution for detection was the DAB reagent (3,3-diaminobenzidine diluted in TBS, pH 7.2 and H_2O_2). Stop solution was 0.03 M H_2SO_4 and then, a final washing (x3) in distilled water and dried. The color intensity of each spot evualated visually.

RESULTS

The Patients

The clinical and diagnostics features of Argentinian patients from whom parasites were studied are presented at Table 1. Eight parasite stocks were successful isolated by culture in Difco Blood Agar (B-45). Less efficient was Schneider's *Drosophila* medium for primary isolation but it supported good growth for bulk promastigotes cultures. Inoculation in hamster was successfull in 50% of isolates.

Both immunodiagnostic tests were positive in all patients from whose parasites were recovered.

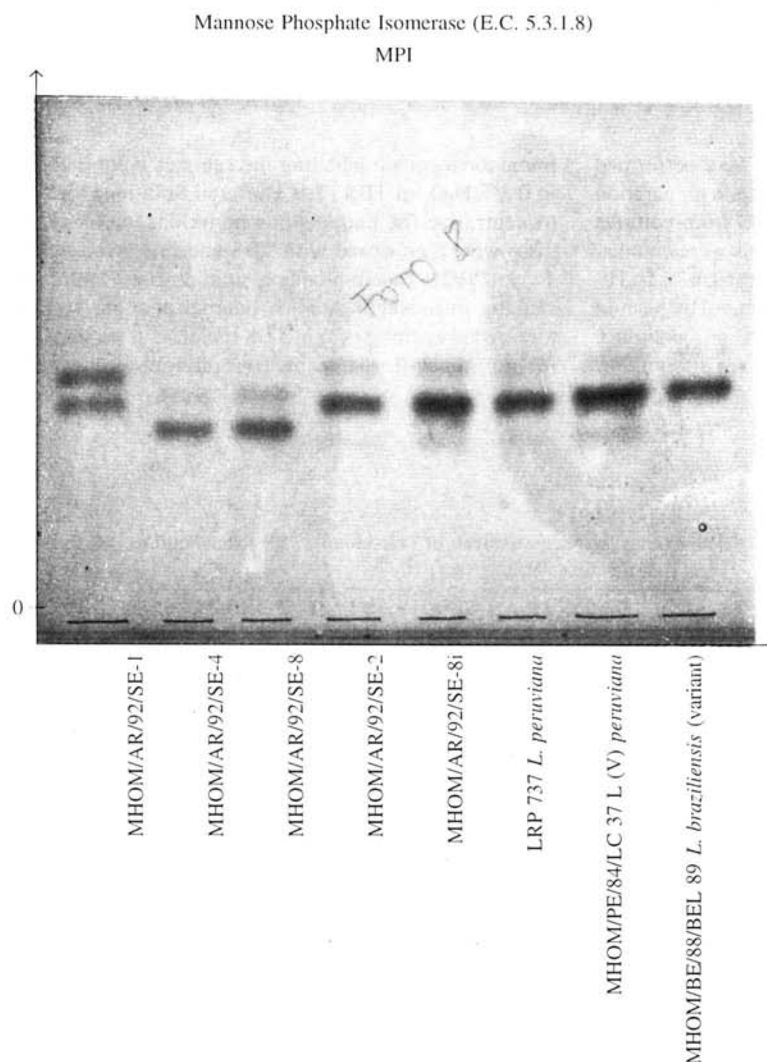

Fig. 3 – Comparison of electrophoretic pattern of MPI of the group of Argentinian stocks with the reference strains, *L. (V) peruviana (lane 6-7)* and *L. braziliensis (lane 8)*.

Parasites

The measurements of parasites (Mean and ± SD) resulted in the following average values in promastigotes: total body length: 24.9 um, ± 3.6; free flagellum: 16.1 um, ± 5; width: 3.5 um, ± 0.9. In amastigotes were: body length: 2.92 um ± 0.53; body width: 1.74 um, ± 0.52.

The most peculiar behaviour in the parasitism of infected hamsters of Argentinian stocks are presented at Table 2. Development of visceral metastasis with intense parasitism with viable amastigotes were detected with some stocks. Lymph node, liver and bone marrow were involved.

Isoenzyme Identification

The five isolates from human cutaneous lesions MHOM/AR/92/SE-1; SE-2; SE-4; SE-8 and SE-8-I had identical bands of enzyme activity to each other and to the WHO *Leishmania (Viannia) braziliensis* reference strain MHOM/BR/83/LTB-300 for the enzymes 6PGD, ES, NH and NH(D) (Figure 2). The same stocks were in enzyme migration pattern different from *L. (Viannia) peruviana* reference strains when tested with the en-

zyme marker of the last specie, MPI. (Figure 3) but not with MDH (not shown).

Monoclonal Antibody Identification

Table 3 summarizes the results of typing with IFAT/MAbs. All eigth stocks showed fluorescence staining reactivity similar to our *L. (V) braziliensis* reference strain MHOM/BR/83/LTB-300. However, three *Leishmania* stocks namely MHOM/AR/92/SE-1; SE-2 and SE-30 did not react with one highly specie-specific Mab (Clone B-18, *L. (V) braziliensis*). Two serodemes were established for the two different antigenic groups: Serodeme I and III. The first is assigned to a group of stocks reacting positive for Mabs: B-2, B-5, B-16, B-17 and B-18 from *Viannia* sub-genus except for B-19, specie-specific *L. (V) guyanensis* monoclonal antibody. Serodeme III, phenotypically characterized by apparently lack of specific epitopes for clone B-18, a highly specie-specific reactive monoclonal antibody marker for *L. (V) braziliensis* parasites, in the IFAT system.

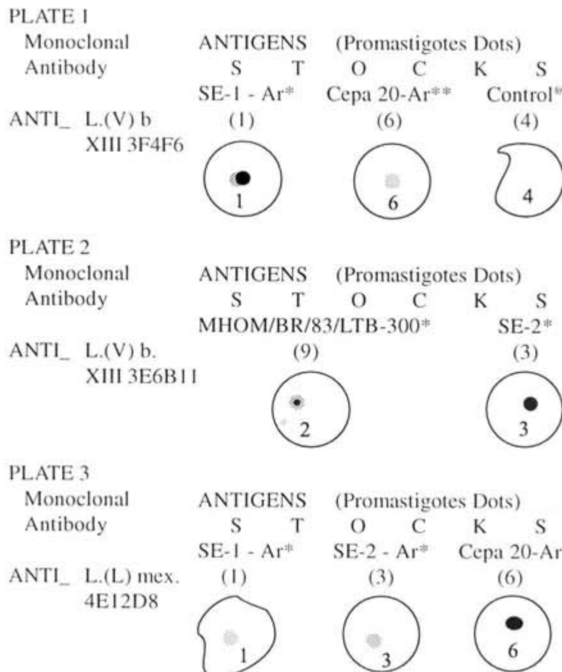

Fig. 4 – Immunodot Assay for *Leishmania* promastigotes identification.
Horseradish Peroxidase Immunoblotting/Monoclonal Antibodies (MAbs) direct membrane binding system.
* Identified as *Leishmania (Viannia) braziliensis* by IFAT/MAbs.
** Identified as *Leishmania (Leishmania) mexicana* by isoenzyme electrophoresis.
@ Antigen+ Peroxidase conjugate.

Figure 4 shows the immunodot assay for identification of promastigotes by a direct binding membrane system. Clear-cut immunostained spots was observed when specific binding is achieved. A representative assay is presented with stocks MHOM/AR/92/SE-1 and SE-2 and MHOM/BR/83/LTB-300, *L. (V) braziliensis*. Similar results (not showed) were obtained with the other stocks.

DISCUSSION

American Mucocutaneous Leishmaniasis (AML) have been reported from the Northern provinces of Argentina by several authors (MAZZA, 1926, 1927, ROMAÑA & ABALOS, 1949; ROMAÑA et al., 1949). According to the Boletin Epidemiologico Nacional (Ministery of Health of Argentina, 1984, 1992, personal communication), AML, have an endemic pattern in most Northern region, specially in Provinces of Salta, Tucuman and Jujuy and more recently, between 1984 and 1986, an epidemic outbreak had been recorded in the Province of Salta and few parasites isolated from human cases were identified as *L. (V) braziliensis*. Further entomological studies by SALOMON et al., (1995) indicate *Lutzomyia intermedia* as potential vector because their predominance and antropophilic behaviour.

A research project was initiated by the Universidad Nacional del Sur, Bahia Blanca and the Hospital Independencia, in the Province of Santiago del Estero aiming the identification of the parasites responsible for the cutaneous lesions of ambulatorial human patients. As recommended by WHO (1990) this is the first step to be accomplished in any endemic area of AML. This will be directly related to the treatment of a patient[11]. From an epidemiological point of view the recognition of the parasites present in the endemic area is fundamental. As far as we know no *Leishmania* parasites have been isolated and identified from the Province of Santiago del Estero.

Size measurements of both amastigotes and promastigotes forms were compatible with *Viannia* leishmanial parasites. Biological, biochemical and immunological parameters of typing demonstrated that Argentinian stocks from the patients studied were *Leishmania (Viannia) braziliensis*. Our finding extend the current described geographical distribution of the most important specie of the *Viannia* sub-genus in South America.

The experimental infections in hamsters with the primary isolates have shown visceral involvement. Metastatic spread to spleen, liver and lymph-nodes following primary cutaneous lesions has been reported by CUBA CUBA et al., (1985) and PETERSON et al., (1988), in Bahia, Brazil but not by TRAVI et al., (1988) with Colombian strains of *L. (V) braziliensis*.

The Difco Blood Agar slant culture system was efficient for primary isolation of parasites in the hospital ambulatorial conditions. These results are in agreement with our previous observations.

Our attempt to reveal the presence of *L. (V) peruviana* amongst Argentinian isolates failed. All five isolates tested with the specific enzyme markers described, MDH and MPI showed unequivocal zymodeme pattern of *L. (V) braziliensis*. It is known that, *L. (V) braziliensis* and *L. (V) peruviana* are genetically very close related. Their zymogrammes differ in only two out of thirteen isoenzymes analyzed[1]. However further studies on many more isolates from the region are necessary in order to elucidate this aspect.

In contrast to homogeneity in the isoenzyme profiles, serodeme analysis by IFAT/MAbs demonstrated two antigenic variants. We ascribed these as serodemes I and III. Serodeme III is characterized by a lack of reactivity to the clone B-18 (XIII 2A5A 10) of monoclonal antibody. This clone has been considered as the most specific and efficient marker for *L. (V) braziliensis* in different endemic regions of America (GRIMALDI et al., 1987). This finding suggest the presence of regional antigenic variants among the *L. (V) braziliensis* strains in Argentina, a fact that deserve further attention.

The direct immunodot assay allowed the relatively rapid and easy identification of *Leishmania* promastigotes by MAbs and a commercially available peroxidase conjugate. It seems that most of the promastigote antigen is bound to the nitrocellulose disc. Similar assays have been described for *Chlamydia trachomatis*[15].

If the goal of a future epidemiologal research is characterize the transmission patterns of the disease in this region of Argentina a full parasite identification is mandatory.

RESUMO

Leishmaniose cutânea humana causada por *Leishmania (Viannia) braziliensis* na Província de Santiago del Estero, Argentina: identificação dos parasitas por anticorpos monoclonais e isoenzimas

Estudos de diagnóstico, caracterização parasitária e identificação foram conduzidos em pacientes humanos com lesões cutâneas de leishmaniose na Província de Santiago del Estero, no Norte da Argentina. Os procedimentos de diagnóstico foram: biópsias de lesões para utilização em esfregaços e inoculação em hamster; aspiração (com agulha) de úlceras, para cultura "in vitro". As técnicas imunodiagnósticas empregadas foram a IFAT-IgG e o teste intradérmico de Montenegro. Oito cepas de parasitas foram isoladas, sendo estas obtidas de pacientes com lesões ativas. Todas as cepas foram inicialmente caracterizadas biologicamente por seu comportamento na infecção experimental do hamster, mensuração dos amastigotas e promastigotas e crescimento "in vitro".

As mesmas oito cepas foram logo identificadas e caracterizadas a nível de espécie, devido a sua reatividade frente a um painel de anticorpos monoclonais subgênero e espécie-específicos. Isso foi realizado utilizando o teste de Imunofluorescência Indireta (IFAT/MAbs) e de um procedimento de Dot-ELISA.

Nós concluímos a partir da análise de serodema dos isolados argentinos que: MHOM/AR/92/SE-1; SE-2; SE-4; SE-8; SE-8-I; SE-30; SE-34 e SE-36 são *Leishmania (Viannia) braziliensis*. Entretanto, três dos isolados de *Leishmania* (SE-1; SE-2 e SE-30) não foram reconhecidos quando testados com um anticorpo monoclonal de reconhecida alta espécie-especificidade (clone B-18, marcador consagrado de *Leishmania (Viannia) braziliensis*), revelando a existência de dois tipos de serodemas entre as cepas estudadas.

Cinco dos oito extratos solúveis de *Leishmania* foram submetidos à eletroforese em gel de amido de camada fina e subseqüentemente examinadas a fim de constatar a atividade das enzimas MPI, MDH, 6PGD, NH, NH-D, SOD, GPI e ES. Fundamentados nos estudos dos corridos eletroforéticos obtidos nos ensaios isoenzimáticos chegamos à conclusão que as cepas MHOM/AR/92 SE-1; SE-2; SE-4; SE-8 e SE-8-I são *Leishmania (Viannia) braziliensis*. É necessário analisar mais enzimas antes de enquadrá-los nos zymodema *braziliensis*.

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