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CLONAL VARIATION WITHIN A MUCOSAL ISOLATE DERIVED FROM A PATIENT WITH *Leishmania* (VIANNIA) *braziliensis* INFECTION.

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SUMMARY

Three isolates over 5 years from a patient with persistent relapsing mucosal leishmaniasis due to *Leishmania* (Viannia) *braziliensis* and 7 clones from one of these isolates were studied by zymodemes and serodemes analysis.

Results showed evidences of clonal phenotypic variation. Eight isoenzymes markers demonstrated clear differences on Cellulose Acetate (CA) and thin starch gel electrophoresis. Also a panel of specific monoclonal antibodies showed such differences.

Our observations provide additional evidence that *Leishmania* (Viannia) *braziliensis* is composed by subpopulations of parasites with peculiar biochemical and antigenic characteristics.

KEY WORDS: *Leishmania* (Viannia) *braziliensis*; Clonal Variation; Zymodemes; Serodemes.

INTRODUCTION

The endemic nature of cutaneous and mucosal leishmaniasis caused by *Leishmania* (Viannia) *braziliensis* in the Três Braços area of Bahia in Brazil is well known and documented in numerous clinical and epidemiological studies (LLANOS-CUENTAS et al., 1984; MARSDEN et al., 1986). During the course of a five-year period of clinical follow-up of a patient with mucosal leishmaniasis with a long history of therapeutic failures 3 separate isolations of the causative organism were made, from one of which cloned populations were derived. This report presents evidence of antigenic and isoenzymic differences between parental and clonal derived organisms isolated from this patient.

MATERIALS AND METHODS

(i) Organisms: Primary isolations of *Leishmania* were made by inoculation of biopsy material into hamsters as described elsewhere (CUBA et al., 1981). Isolation were made in July 1982 after treatment of the patient with Glucantime; in De-

cember 1985 after recurrence of the lesion, and finally in March 1987. The patient M.B. (LTB-12) has had relapsing mucosal leishmaniasis for 15 years. He improves on treatment with antimonials, Amphotericin B or Pentamidine only to relapse subsequently with granulomas of the nose, palate, pharynx and larynx (MARSDEN et al., 1985).

(ii) Primary cultivation "in vitro". Material from the lesions in the hamsters was inoculated onto Difco Blood Agar slopes USAMRU (WALTON et al., 1977) and leishmanial promastigotes cryopreserved from the 2nd and 3rd subcultures.

When the primary cultures in Difco Blood Agar were established, passages were made in Schneider's *Drosophila* Medium supplemented with Foetal Calf Serum (FCS) and MEM: FCS: ELB Medium (EVANS et al. 1984). The pellet of organisms produced was treated in order to yield a supernatant containing the soluble enzymes of the *Leish-*

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mania (LE BLANCQ et al., 1986 (a)). These supernatants were then examined immediately or stored in bead form under liquid nitrogen until required.

The WHO reference strains of *Leishmania* used in this work were obtained from WHO Reference Centre Cryobank, London School of Hygiene and Tropical Medicine, Winches Farm, St. Albans, England (*L. (V) braziliensis*, MHOM/BR/75/M-2903, MHOM/BR/75/M-2904, (MHOM/BR/83/LTB-300); *L. (V) guyanensis*, MHOM/BR/75/M-4147 and *L. (V) panamensis*, MHOM/PA/71/LS-94).

Cloning of the isolates was done by the single cell isolation procedure as described by DVORAK (1985). Clones derived from the LTB-12-December-1985 - isolate are called "recrudescence clones" and coded C-I; C-III; C-VI; C-VII; C-VIII; and C-X. Fresh Schneider's *Drosophila* Medium supplemented with 20% FCS was used for mass production of parasite cultures.

In our laboratory at Brasilia University, initially 5 enzymes were examined by Cellulose Acetate (CA) electrophoresis and enzyme development following the methodology of KREUTZER & CHRISTENSEN, 1980. The enzymes were: Glucose Phosphate Isomerase (GPI E.C. 5.3.1.9); Phosphoglucomutase (PGM E.C. 2.7.5.1); Glucose-6-phosphate dehydrogenase (G-6-PD E.C. 1.1.1.49); Mannose Phosphate Isomerase (MPI E.C. 5.3.1.8); and Malic Enzyme (ME E.C. 1.1.1.40).

Furthermore, eighteen months at the WHO Reference Centre in London, 12 enzymes were examined by thin layer starch gel electrophoresis using basically the methods described by EVANS et al., 1984. The following enzymes were examined: Malate Dehydrogenase (MDH E.C. 1.1.1.37); Alanine Aminotransferase (ALAT E.C. 2.6.1.2); Aspartate Aminotransferase (ASAT 2.6.1.1); Glucose Phosphate Isomerase (GPI E.C. 5.3.1.9); Phosphoglucomutase (PGM E.C. 2.7.5.1); Nucleoside Hydrolase (NH 3.2.2.2); B-Esterase (ES E.C. 3.1.1.1.1); Mannose Phosphate Isomerase (MPI E.C. 5.3.1.8); Phosphogluconate Dehydrogenase (6PGD E.C. 1.1.1.44); Proline Imino-peptidase (PEP-D E.C. 3.4.11.5); Superoxide Dismutase (SOD E.C. 1.1.5.1.1); and Pyruvate Kinase (PK E.C. 2.7.1.40).

The electrophoretic conditions and stains for ALAT, ASAT, 6PGD, GPI, MDH, MPI, PGM and

SOD were those given by EVANS et al., (1984). The conditions for PK, ES, NH and PEP-D were basically those as given by LE BLANCQ et al., (1986 b). Samples were applied in groups of eight or ten per plate and were compared to each other and to the WHO reference strains of *L. (V) braziliensis*, MHOM/BR/75/M2904, MHOM/BR/83/LTB-300; *L. (V) guyanensis*, MHOM/BR/75/M4147 and *L. (V) panamensis*, MHOM/PA/71/LS-94.

The Indirect Immunofluorescent antibody test with promastigotes was used in these studies as described by Mc MAHON PRATT et al., (1986) against a panel of group and species specific monoclonal antibodies. Briefly, parasites promastigotes were placed on the slide by using a Shandon Elliot centrifuge (Surrey, England), air dried fixed with methanol, or not fixed and covered with PBS, 1% Bovine serum albumin (PBS-BSA). Samples were covered with appropriate dilution of each monoclonal antibody. Three assays were made at 1: 1000 routine working dilution of hybridoma ascites containing the monoclonal antibody. The slides were rinsed with PBS-BSA and then incubated with Fluorescein-Conjugate rabbit Antimouse antisera (Jackson Research, England) at 1: 20 dilution after centrifugation to remove undiluted fluorescein. Slides were washed, mounted and examined using fluorescence microscope.

RESULTS

Our attempts at cloning promastigotes resulted in the successful establishment of 7 clones using the single cell isolation procedure. All three original isolates of LTB-12 July 82, December 85 and March 87 and the seven clones derived from LTB-12 December 85 were examined. Clear reproducible resolution was obtained using the thin starch gel electrophoresis system. CA electrophoresis was less efficient but G6PD was clear enough (results not shown) to allow us to visualize clear variations patterns with clones C-III and C-VI when compared with the five other clones and the original isolate.

Figure I presents the enzymes which show differences among the parental isolate and clones. The actual gels for GPI, PGM, 6PGD and PEP-D are shown in Figure 2. All these enzymes showed clearcut differences between clones C-III and C-VI and the five related clones, parental LTB-12 December 85 and WHO references strains. The two clones (C-III and C-VI) seem to be similar each to

other in most of their profile spectrum but with slight differences in G6PD in which clone C-III shows two bands. Four enzymes, NH, ES, PK and MDH gave the same banding pattern for all isolates and clones. Meanwhile, MPI, SOD, GPI and 6PGD each had multibanded among parental and clone populations.

When the clones C-III and C-VI were compared with the reference strains of *L. (V) panamensis* and *L. (V) guyanensis* by the enzyme ALAT, ASAT, MPI, PGM, GPI, PEP-D, they displayed enzyme phenotypes that distinguish them from the reference strains (Figures 3 and 4). Both clones were also compared with three isolates recovered from the same patient in 1982, 1985 and 1987. The enzyme profiles of the three original isolates were similar and showed clear differences from those of the clones (Figure 4).

Table I shows results based on an indirect immunofluorescence assay by monoclonal antibodies. C-III and C-VI demonstrated antigenic differences because they were found to be negative for all the battery of reagents. None of the clones reacted with the *L. (V) guyanensis* specific monoclonal.

DISCUSSION

LOPES et al., 1984 and PACHECO et al., 1986, detected the presence of subpopulations of different schizodemes in isolates of New World *Leishmania* and latter workers recorded mixed infections by using cell cloning techniques. Clones from an original human isolate showed two distinct schizodemes indicating the presence of two different populations of *Leishmania*.

Research on *L. (V) braziliensis* variability at the clonal level has just been initiated. This problem is important because there may be temporal changes in strains, population virulence and pathogenicity to the host as shown by the history of the patient investigated here (LTB-12). Our results shown that real differences exist among clones of *L. (V) braziliensis* in the enzymes ASAT, ALAT, SOD, 6PGD, PEP-D, MPI, GPI, PGM and G6PD (this last not shown); this may reflect genetic variability.

Two clones out of 7 derived from the same parental isolate revealed isoenzyme heterogeneity. The zymodeme of both recrudescient clones did not fit the known zymodemes recognized in the same

Figure 1
Diagrammatic representation of enzyme polymorphism distinguishing zymodemes of clones C-III and C-VI from LTB-12 (85) parental isolate, other clones derived from the latter and W.H.O. reference strains for *Leishmania (V) Braziliensis*.

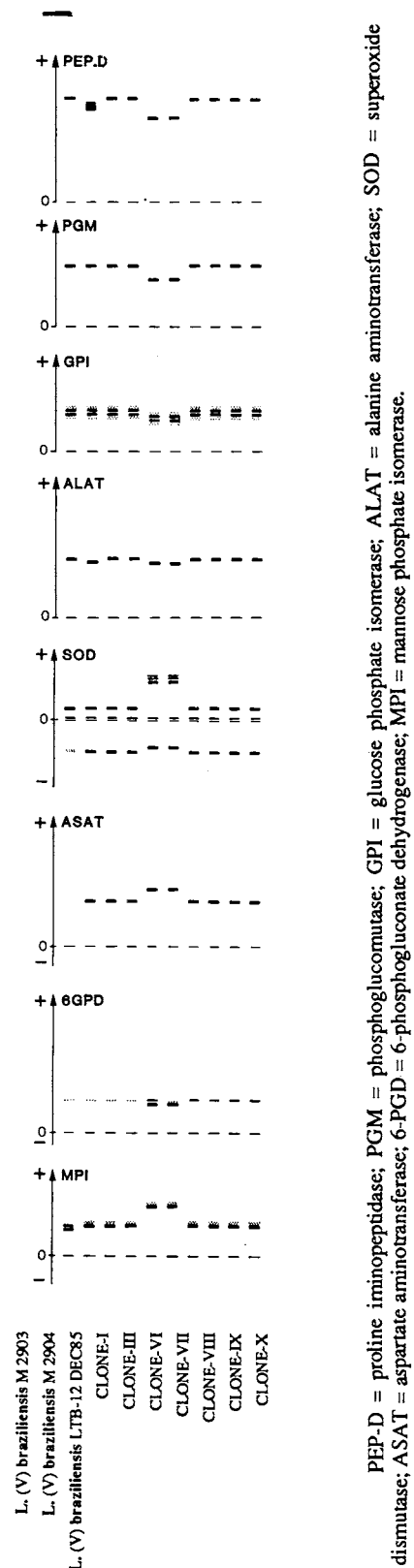


Figure 2

Photographs of the electrophoretic patterns obtained with soluble extracts of cultured *Leishmania* promastigotes for 4 enzymes: GPI, 6PGD, PGM and PEP-D in thin starch gel electrophoresis-LTB-12(85), clones derived from LTB-12 and W.H.O. reference strains of *Leishmania (V) Braziliensis*.

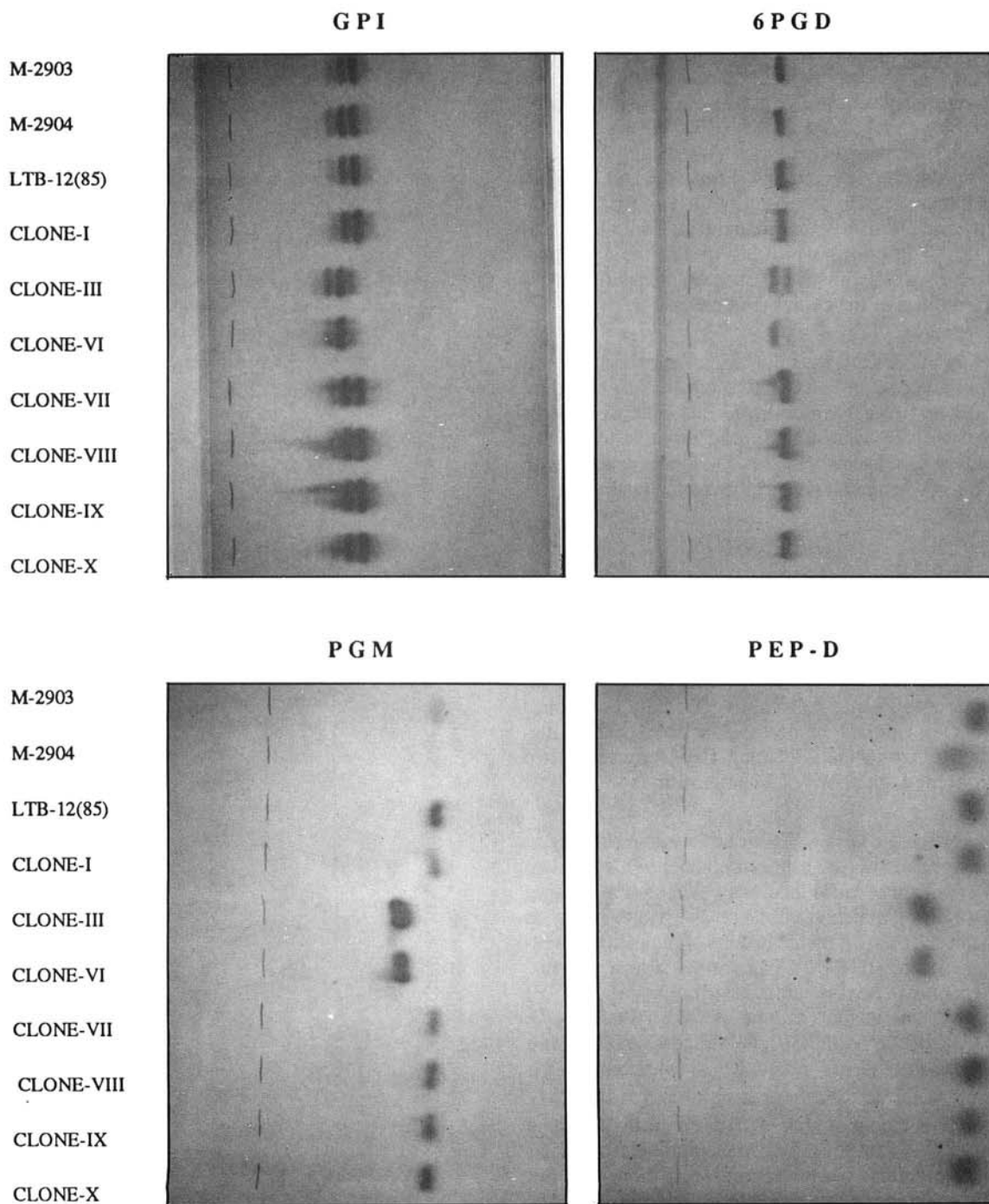


Figure 3

Photographs of the electrophoretic patterns obtained with soluble extracts of cultured *Leishmania* promastigotes for 3 enzymes: MPI, PEP-D and PGM in thin starch gel electrophoresis. Comparison among parental isolate LTB-12(85), clones C-III and C-VI derived from the latter and W.H.O. reference strains of *L. (V) Braziliensis*, *L. (V) Guyanensis* AND *L. (V) Panamensis*.

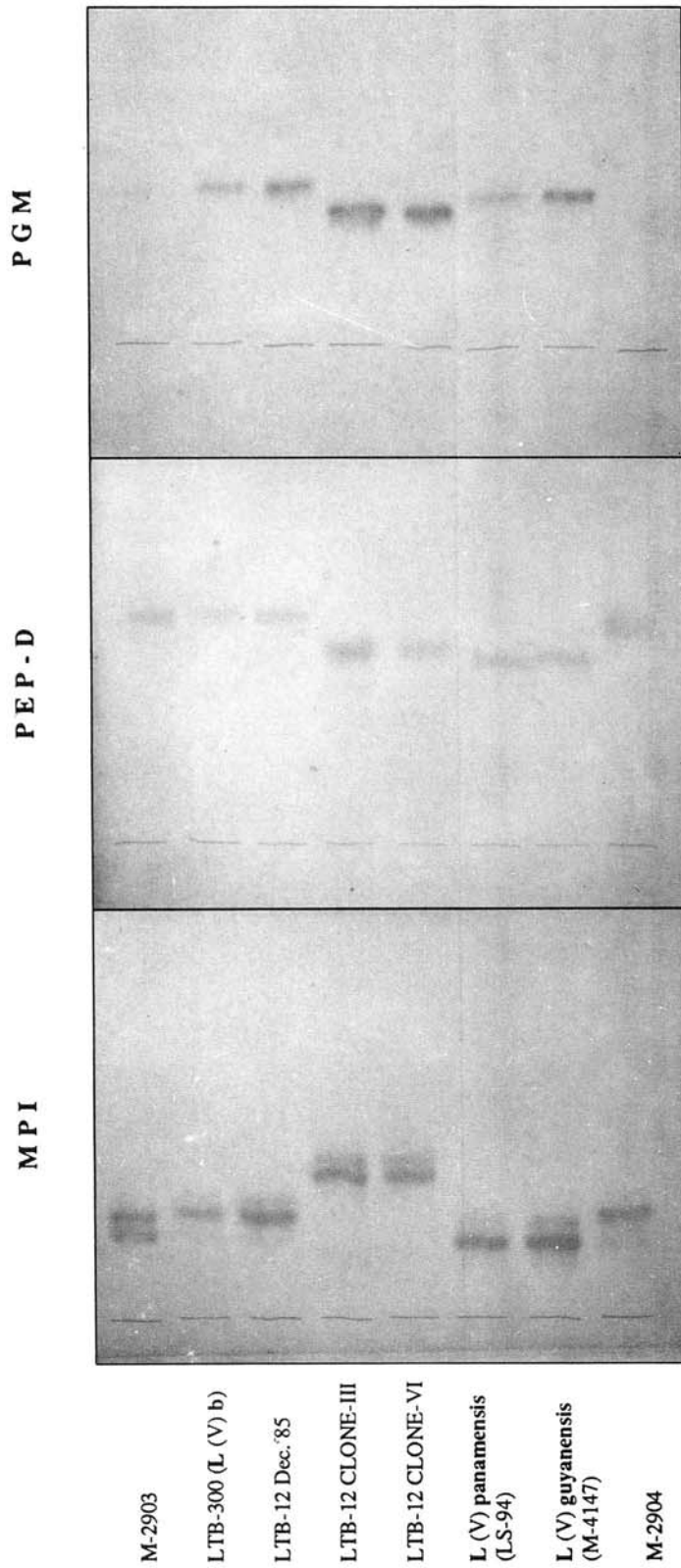


Figure 4

Photographs of the electrophoretic patterns obtained with soluble extracts of cultured *Leishmania* promastigotes for 3 enzymes, ASAT, MPI and PGM in thin starch gel electrophoresis. Comparison among parental isolate LTB-12 (85), clones C-III and C-VI derived from the latter, isolates LTB-(82), LTB-12(87) and W.H.O. reference strains of *L. (V) Braziliensis*.

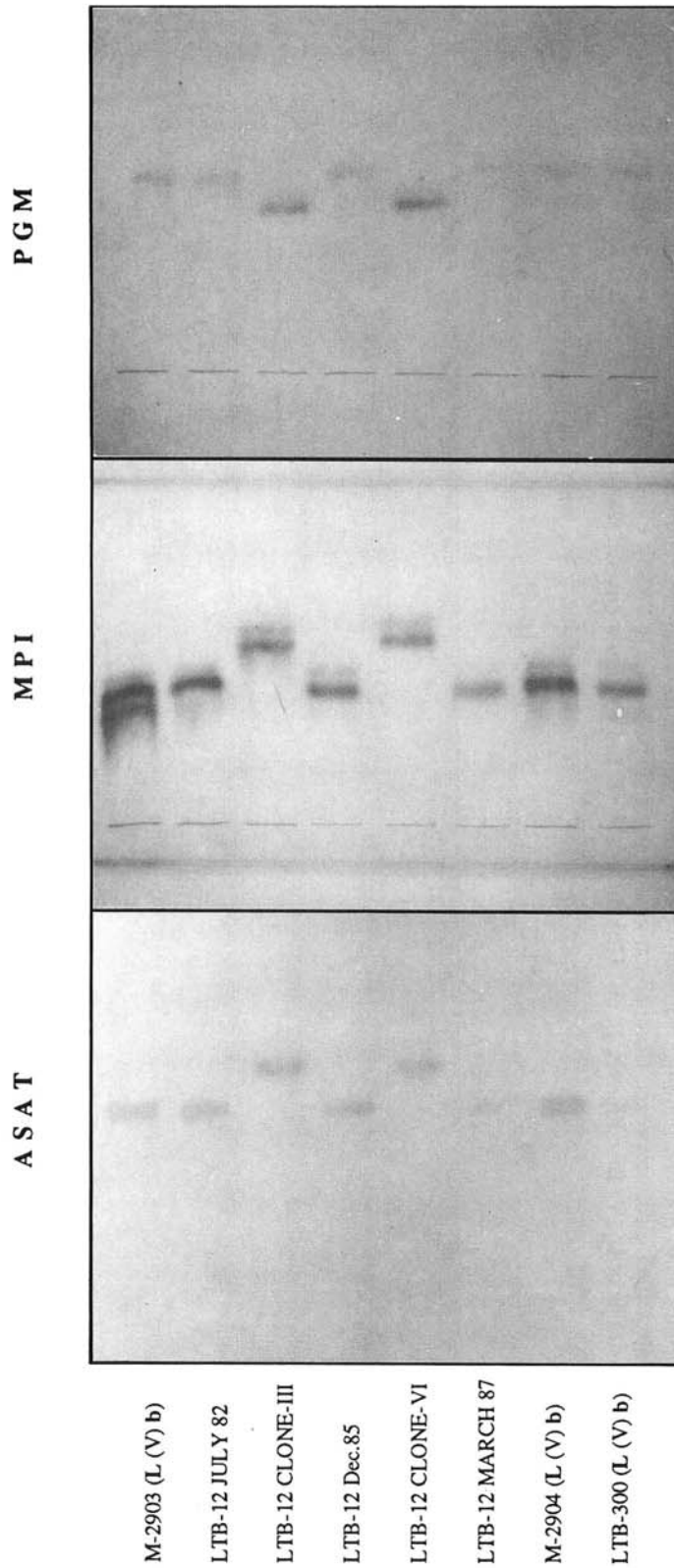


TABLE 1

Antigenic characterization of clones produced from isolate MHOM/BR/85/LTB-12-Dec-85 of *Leishmania* (*VIANNIA*) *Braziliensis** by IFAT

MONOCLONAL ANTIBODIES CODE**	SPECIFICITY	ORIGINAL UNCLONED ISOLATE	REFERENCE N° OF CLONES DERIVED FROM PARENTAL ISOLATE						
			C-I	C-III	C-VI	C-VII	C-VIII	C-IX	C-X
VI4B9 D10 B-2	L.(<i>Viannia</i>)	V***	—	—	—	+	V	—	—
XIII 3FAF6 B-17	L. (V) <i>braziliensis</i>	+	+	—	—	+	+	+	+
XIII 2A5A10 B-18	L. (V) <i>braziliensis</i>	+	+	—	—	+	+	+	+
XIII 3E6B11 B-16	L. (V) <i>braziliensis</i>	+	+	—	—	+	+	+	+
VII 2C5C5 B-5	L. (<i>Viannia</i>) <i>braziliensis</i>	+	+	—	—	+	+	+	V
XLIV 5A2B9 B-19	L. (V) <i>guyanensis</i>	—	—	—	—	—	—	—	—
IX 2H7E10 M-2	L. (<i>Leishmania</i>) <i>amazonensis</i>	—	—	—	—	—	—	—	—
LXVIII 4E12D8 M-12	L. (L) <i>mexicana</i>	—	—	—	—	—	—	—	—
IX 1F9D8	L. (L) <i>amazonensis</i>	—	—	—	—	—	—	—	—
LXVII 1D7B8	L. (L) <i>mexicana</i>	—	—	—	—	—	—	—	—

* Isolated from LTB-12, patient with mucosal granuloma, Três Braços, Bahia - December 1985.

** Tested at 1:1000 working dilution, except B-2 (1/500), three different assays each antibody.

*** V = Variable, presence or absence of reaction.

endemic area (Cuba, unpublished) and parental isolate did not display any variation with the same 13 enzymes analysed.

It would be mentioned that the electrophoretic system of thin starch electrophoresis is sufficiently sensitive and covers a wide range of variation to distinguish heterogeneity among clones of a single strain. In addition, is interesting to note the stability of the enzyme clonal variants because our previous studies carried out eighteen months ago using CA electrophoresis had already detected this enzymic behaviour with a few enzymes (G6PD, MPI, MPI and ME, results not shown).

The serodeme analysis by monoclonal antibodies showed total lack of reactivity of the same two clones with a distinct zymodeme. The reason

for this apparent lack of binding could be either because they do not have the antigenic determinant (s) or these have too low an epitope density for immunofluorescence assay. In fact, as reported by Mc MAHON-PRATT et al., (1984) stocks of *Leishmania* that have been found to be apparently negative by immunofluorescence have been determined subsequently to be definitely positive by Indirect Radioimmune assay (RIA). This reflects the poorer sensitivity of the former method. However, SARAVIA et al., (1985) have noticed one mucosal isolate of *L. (V) braziliensis* that appears to be null for the subspecies determinants detected by monoclonal antibodies/RIA. The coincidence of these differences in systems of characterisation utilised here (isoenzymes and serodeme typification) indicate that heterogeneity occurs among the clones of a single strain of *L. (V) braziliensis*.

The degree of heterogeneity with other isolates from the same endemic area and its correlation with other biological characteristics requires more detailed investigation. Both variability and immunocompetence of the host and differences in *Leishmania* parasites may influence the course of diseases. It is possible that a subpopulation of *L. (V) braziliensis* could be the causative agent of mucocutaneous leishmaniasis.

Our observations provide evidence that *L. (V) braziliensis* is composed by subpopulations of parasites with certain biochemical and immunological characteristics. Experiments applying the resolving power of Pulse Field Gradient gel electrophoresis to analyse the genomic organization of the isolates and clones are in progress.

RESUMO

Varição clonal de um isolado derivado de um paciente com infecção mucosa pela *Leishmania (Viannia) braziliensis*

No transcurso de um período de 5 anos foram estudados 3 isolados de um paciente com leishmaniose mucosa recidivante causada pela *Leishmania (Viannia) braziliensis* e 7 clones de um desses isolados. Este estudo foi feito pela análise dos serodemas e zimodemas.

Os resultados indicaram a ocorrência de variações fenotípicas clonais. Oito marcadores isoenzimáticos demonstraram diferenças nos padrões eletroforéticos em Acetato de Celulose (AC), bem como em camada fina de amido. Da mesma forma foram constatadas diferenças em um painel de anticorpos monoclonais específicos e subespecíficos.

Nossas observações indicam ainda que a *Leishmania (Viannia) braziliensis* está composta por subpopulações de parasitas com características bioquímicas e antigênicas peculiares.

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