CLIN. CHEM. 30/12, 2048–2052 (1984)

Two-Dimensional Electrophoresis Used to Differentiate the Causal Agents of American Tegumentary Leishmaniasis

Nancy G. Saravia,[1] M. Anne Gemmell,[2] Sharon L. Nance,[2] and N. Leigh Anderson[2]

American tegumentary leishmaniasis is caused by a diverse group of *Leishmania* classified within two species complexes: *L. mexicana* and *L. braziliensis*. Because distinct disease forms are associated with certain species or subspecies, prognosis requires taxonomic identification of the pathogen. Biological criteria allow identification only to the species level; thus, biochemical and immunological markers are eagerly sought. We have used two-dimensional electrophoresis to examine the relationships among reference strains of New World *Leishmania* and stocks isolated from Colombian patients. The *L. mexicana* and *L. braziliensis* species complexes are shown to be extremely disparate, and the relative affinities of the subspecies reference strains and Colombian *Leishmania* stocks are documented. The latter displayed a variety of patterns—some clearly identifiable with a particular *L. braziliensis* subspecies reference strain, others intermediate between those of *L.b. panamensis* and *L.b. guyanensis*. Such comparisons are useful both in establishing relatedness and identifying subspecies and variant marker proteins, which may have biological significance.

Additional Keyphrases: *phenotypic relationships · parasitology · electrophoresis, two-dimensional*

Dermal leishmaniasis, caused by the flagellated protozoa *Leishmania*, belonging to the order Kinetoplastida, family Trypanosomatidae, is both common and widespread in Central and South America and the Caribbean (*1*). Autochthonous infections also occur, though rarely, in Texas (*2*). The frequently protracted nature of the lesions and the potential for grossly disfiguring disease expression render this zoonotic agent an important public-health problem. Geographic origin and disease form have traditionally been utilized as distinguishing criteria for the species of *Leishmania* infecting humans (*3, 4*). Nevertheless, the multifactorial basis of the latter parameter and the overlapping distribution of what are now recognized as the *Leishmania mexicana* and *Leishmania braziliensis* species complexes limit the reliability with which the parasites can be identified based on these criteria.

Biological studies have provided a behavioral basis for discriminating between the *L. mexicana* and *L. braziliensis* complexes: growth kinetics in culture (*5*), localization during development in the gut of the sandfly vector (*6*), and the evolution of infection in experimentally inoculated golden hamsters distinguish these two groups (*3*). However, because the subspecies that constitute the complexes are not separable by these criteria, yet appear to display different pathogenic capabilities—latency, metastasis, and drug resistance—intrinsic markers have been sought. The various approaches explored include comparisons of the buoyant density of nuclear and kinetoplast DNA (*7–9*), radio-respirometry (*10*), immunological identity of factors excreted by parasites grown in culture (*11*), hybridization of DNA probes with kinetoplast DNA (*12, 13*), electrophoretic separation of enzyme variants (*14–16*), and antigenic discrimination by use of monoclonal antibodies (*17, 18*).

Although each of these methods contributes to an overall understanding of the heterogeneity of *Leishmania*, all of them examine a very small portion of the genetic composition or phenotypic profile of these organisms. Two-dimensional electrophoresis permits the separation and simultaneous observation of hundreds of gene products and, therefore, of multiple electrophoretically detectable polymorphisms. Because taxonomic relationships as well as the definition of phenotype markers are most adequately established in the context of a constellation of characters, we have explored the usefulness of two-dimensional electrophoresis in determining relatedness among selected World Health Organization reference strains and *Leishmania* recently isolated from patients. Our preliminary observations, reported here, provide striking and quantifiable evidence of the disparity between *L. mexicana* and *L. braziliensis* complexes, and shed new light on the phenotypic relationships among the constituent subspecies.

Materials and Methods

Reference Strains of *Leishmania*

The following *Leishmania* strains, selected by the World Health Organization as reference prototypes (*1*), were obtained from the cryobank of the London School of Tropical Medicine and Hygiene and used in this study:

Subspecies of the *L. braziliensis* complex:	
L. braziliensis braziliensis	HOM/BR/75/M2903
L. braziliensis guyanensis	HOM/BR/75/M4147
L. braziliensis panamensis	HOM/PA/71/LS94
Subspecies of the *L. mexicana* complex:	
L. mexicana mexicana	NYC/BZ/62/M379
L. mexicana amazonensis	FLA/BR/67/PH8
L. mexicana pifanoi	HOM/VE/57/LL-1

Colombian Isolates

The parasites were isolated by syringe aspiration or biopsy of lesions in patients seeking diagnosis and treatment for tegumentary ulcers compatible with leishmaniasis. The material so obtained was inoculated into diphasic blood agar medium (*5*) and subcutaneously into the nose of golden hamsters. The hamster is highly susceptible to infection by these organisms, and provides a means of isolating *Leishmania* from secondarily infected tissues.

Culture Conditions

Leishmania to be studied were transferred from the liquid overlay of blood agar medium to Schneider's *Drosophila* medium (GIBCO, Grand Island, NY 14672), containing 100 mL of heat-inactivated fetal calf serum and 10 mL of

[1] Centro Internacional de Investigaciones Médicas (CIDEIM), Tulane University—Colciencias, Apartado Aéreo 5390, Cali, Colombia.

[2] Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439.

Received July 17, 1984; accepted September 4, 1984

penicillin/streptomycin mixture (GIBCO) per liter. Cultures were maintained at 27 °C for three to five days.

Preparation of *Leishmania* Extracts

Promastigotes were harvested during exponential growth by centrifugation at 1000 × *g* at 4 °C for 10 min. Cell pellets were resuspended in 400 μL of Dulbecco's phosphate-buffered saline and transferred to Eppendorf microcentrifuge tubes. The suspension was centrifuged for 5 s at 2000 × *g* in a Beckman Microfuge, the supernate was aspirated, and the packed cells were solubilized in a solution containing, per liter, 9 mol of urea, 20 g of NP-40 detergent, 20 g of ampholytes (Ampholines; LKB, Gaithersburg, MD 20877), and 10 g of mercaptoethanol (*20*).

Two-Dimensional Electrophoresis

To resolve the solubilized proteins by two-dimensional electrophoresis, we used an adaptation of the ISO-DALT system (*21, 22*) for acidic and neutral proteins that is currently being developed at Argonne National Laboratory. The slab-gel size has been increased from 17.5 × 17.5 cm to 20.5 × 25.5 cm, and gels are prepared by a computer-controlled slab-gel maker. The 24-cm ISO gels used in the first-dimension separation contained, per liter, the following ampholytes: 600 mL of Pharmalyte 5-8 (Pharmacia Fine Chemicals, Piscataway, NJ 08854), 200 mL of Pharmalyte 3-10, and 200 mL of Servalyte 3-10 (Serva Fine Biochemicals, Heidelberg, F.R.G.). Linear gradient sodium dodecyl sulfate-slab gels (acrylamide 100–200 g/L) were used for the second-dimensional separation. Gels were fixed in an equivolume mixture of ethanol and water containing 1 mL of formaldehyde per liter, then silver stained (*23*).

Results

Comparison of the two-dimensional patterns obtained for the *L. mexicana* subspecies with those of *L. braziliensis* subspecies revealed a noteworthy lack of co-migrating proteins other than residual bovine serum components. Figure 1 illustrates the distinctive patterns observed for the two species. In contrast, patterns for subspecies within each complex were remarkably similar. Within the *L. mexicana* complex, *L.m. mexicana* and *L.m. pifanoi* reference strain patterns were practically indistinguishable, but *L.m. amazonensis* showed several distinctive protein spots (Figure 2). The three subspecies reference strains of the *L. braziliensis* complex studied were separable on the basis of their two-dimensional patterns; as shown in Figure 3, *L.b. panamensis* and *L.b. guyanensis* are more closely matched with each other than with *L.b. braziliensis*. Charge shifts appear to account for most of the differences within species.

Comparison of organisms recently isolated from lesions of Colombian patients with the subspecies reference strains confirmed that all those studied thus far belong in the *L. braziliensis* species complex, as was indicated by their biological behavior and isoenzyme phenotypes. Furthermore, their two-dimensional-gel patterns could be grouped into at least three categories: (*a*) variants of *L.b. braziliensis* (Figure 4); (*b*) strains identical to the *L.b. panamensis* reference prototype (Figure 5); and (*c*) variants intermediate between *L.b. panamensis* and *L.b. guyanensis* (Figure 6). The Colombian variants of *L.b. braziliensis* are distinguishable from the Brazilian reference strain and from each other on the basis of several differences in the two-dimensional patterns. For example, the boxed area on patterns for isolates 71 and 87 in Figure 4 delineates spots not observed in either the reference strain or the other Colombian variant pictured. The category defined as intermediate between *L.b. panamensis* and *L.b. guyanensis* consists of isolates whose

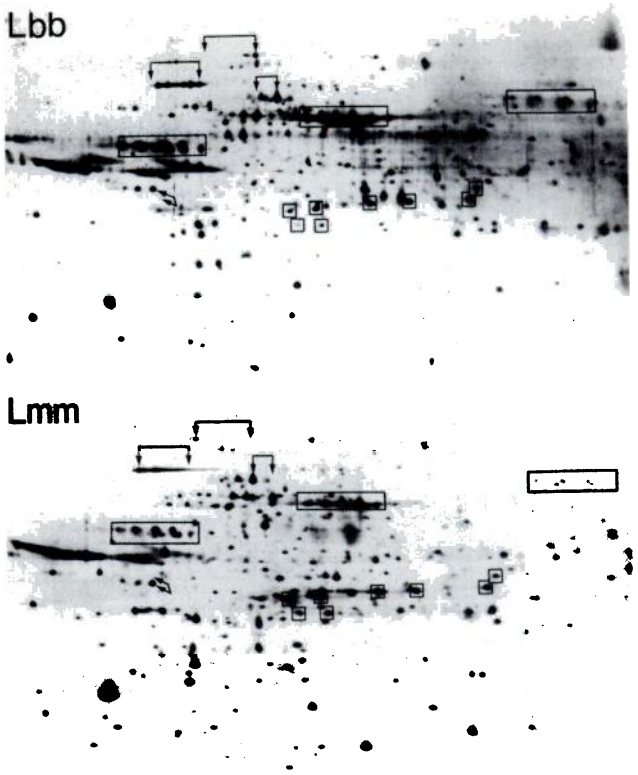

Fig. 1. Comparison of the *L. braziliensis* and *L. mexicana* species complexes

Boxes indicate fetal-calf serum proteins, which provided internal position standards and were used to register patterns being compared. Some of the major probable charge shifts are indicated by *arrows*. Detailed comparisons, undertaken by overlaying these patterns, show that there are few, if any, co-migrating proteins among the several hundred *Leishmania* polypeptides examined. This suggests that one or more charge–change mutations have occurred in each gene since these species diverged

patterns are not identical to either prototype, but are clearly closer to the *L.b. panamensis/L.b. guyanensis* phenotype than to the *L.b. braziliensis* phenotype. Within this intermediate group, individual isolates appear to align more closely with one of either the *L.b. panamensis* or *L.b. guyanensis* reference strains, as shown in Figure 6, which illustrates a portion of the corresponding two-dimensional patterns. However, the overlap of markers detected in the patterns for *L.b. guyanenis* and *L.b. panamensis* reference strains and those of the Colombian isolates discouraged our designation as variants of either subspecies.

We also examined metabolically labeled extracts prepared from some of these *Leishmania,* although these data are not included. The patterns we observed corroborate the results obtained by silver staining. Autofluorography after labeling with [^{35}S]methionine gives greater resolution, and has allowed us to identify additional putative subspecies and strain-specific markers.

Discussion

The degree of disparity between the *L. mexicana* and *L. braziliensis* patterns was unexpected. Although isoenzyme phenotyping (*14–16*) and, more recently, cross-hybridization studies of kinetoplast DNA (*12, 13*) have established the divergence of these two species, the magnitude of the phenotypic differences at the molecular level—directly or indirectly attributable to differences in genomic DNA—was previously undocumented. The affinities (i.e., the similarities) between particular subspecies within each complex are clearly demonstrable by two-dimensional electrophoret-

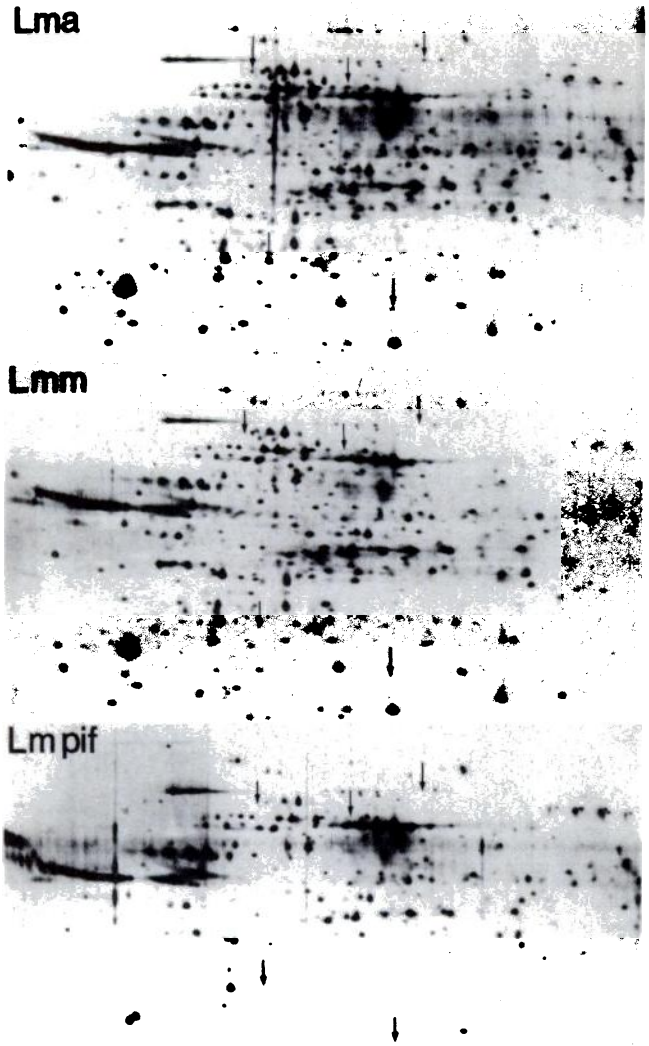

Fig. 2. Comparison of the subspecies within the *L. mexicana* complex
Arrows indicate protein spots present in *L. m. amazonensis* (*Lma*) but apparently absent from *L. m. mexicana* (*Lmm*) and *L. m. pifanoi* (*Lmp*). *Lmm* and *Lmp* are extremely similar

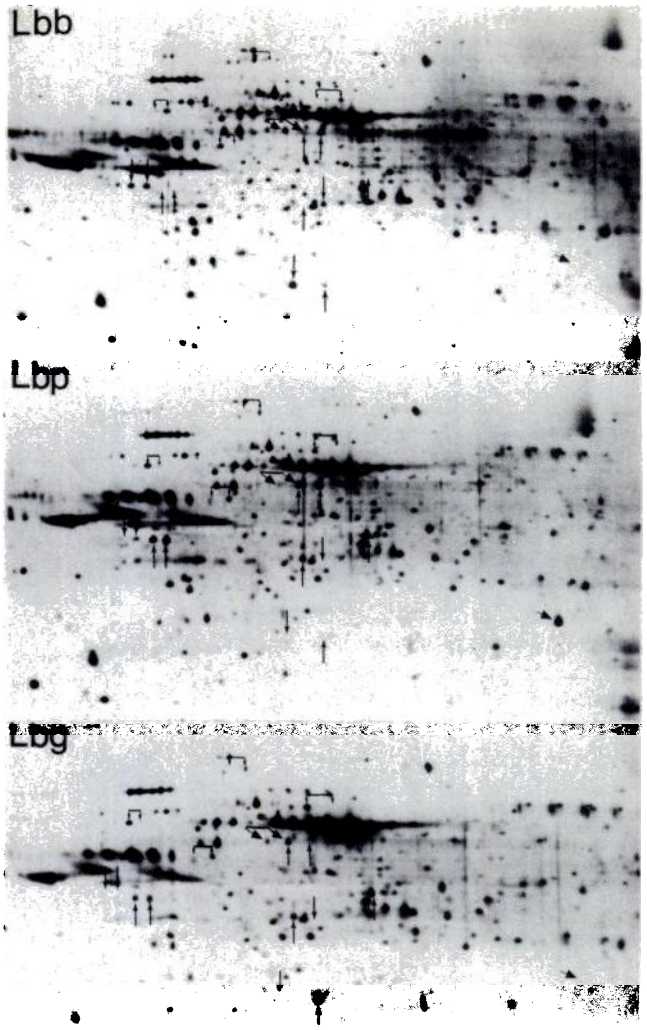

Fig. 3. Comparison of three subspecies within the *L. braziliensis* complex
Arrows indicate protein spots that distinguish *L. b. braziliensis* (*Lbb*) from *L. b. panamensis* (*Lbp*) and *L. b. guyanensis* (*Lbg*). Most of these are interpretable as charge-change variants, consistent with single base mutations in the DNA. A putative marker for *L. b. panamensis* is indicated by the *arrow* in the right lower quadrant

ic analysis, if we assume the validity of the reference material. Thus *L.m. pifanoi* and *L.m. mexicana* very closely approximate one another, while *L.m. amazonensis* diverges from the other two. A similar dichotomy is evident in the *L. braziliensis* complex, with *L.b. guyanensis* and *L.b. panamensis* merging through a spectrum of related variants and *L.b. braziliensis* diversifying away from the former cluster.

The identity of an infectious agent comprehends its relatedness to similar organisms as well as its uniqueness. Differences and similarities are most adequately examined in the broad context of multiple traits. The number and variety of gene products that can be examined by two-dimensional electrophoresis should provide a more nearly complete—and consequently more nearly accurate—appraisal of identifying characteristics.

Two-dimensional electrophoresis should not be routinely required for the identification of *Leishmania;* rather, it provides a reliable basis for validating reference strains and the more readily used marker systems, such as electrophoretically detectable variants and antigenic determinants detected by monoclonal antibodies. We have found that the interpretation of isolated results obtained by these latter methodologies can be discrepant, probably because of the still-undefined heterogeneity of these organisms, as well as the small number of markers or gene products studied. Standardization of isoenzyme and monoclonal-antibody panels in accordance with guidelines prompted by two-dimensional protein patterns could diminish, if not obviate, current ambiguities. However, such standardization will require the examination of a larger number of reference strains and stocks to delineate the range of variation within taxa.

This report demonstrates the utility of two-dimensional electrophoresis in defining relatedness among *Leishmania.* A more complete understanding of the taxonomic and biological inter-relationships of these parasites appears to be achievable through the exploitation of this powerful method of protein resolution. Moreover, interpretation of markers in the context of biological, epidemiological, and clinical aspects of each stock may reveal functionally significant associations that will contribute to a better understanding of parasite determinants of pathogenesis.

This research was supported in part by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, and in part by the U.S. Department of Energy under contract W-31-109-ENG-38.

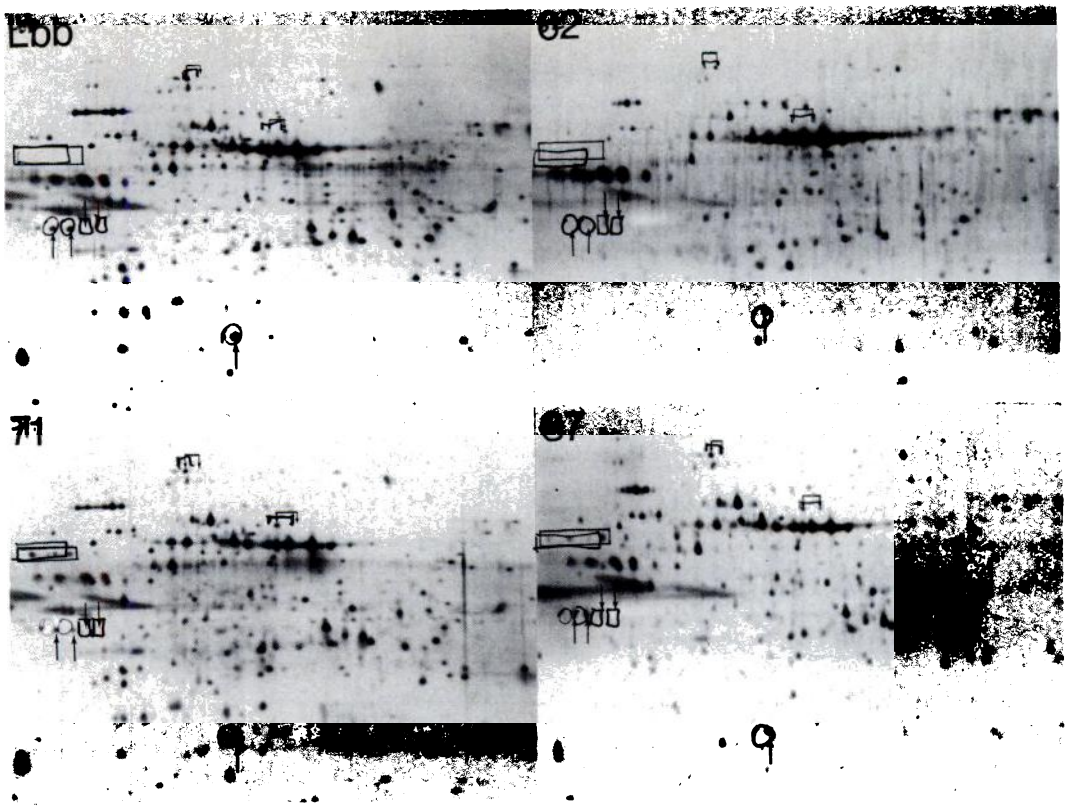

Fig. 4. Two-dimensional patterns of a *L. b. braziliensis* (*Lbb*) reference strain and Colombian variants

Box encloses area defining a set of protein spots found in Colombian *Lbb* variants 71 and 87 but not in variant 62 or the reference strain. *Arrows* indicate putative subspecies-specific markers and some selected charge shifts

Fig. 5. Two-dimensional patterns of *L. b. panamensis* (*Lbp*) reference strain and apparently identical Colombian isolate

Possible Lbp marker proteins are indicated by the *arrows*

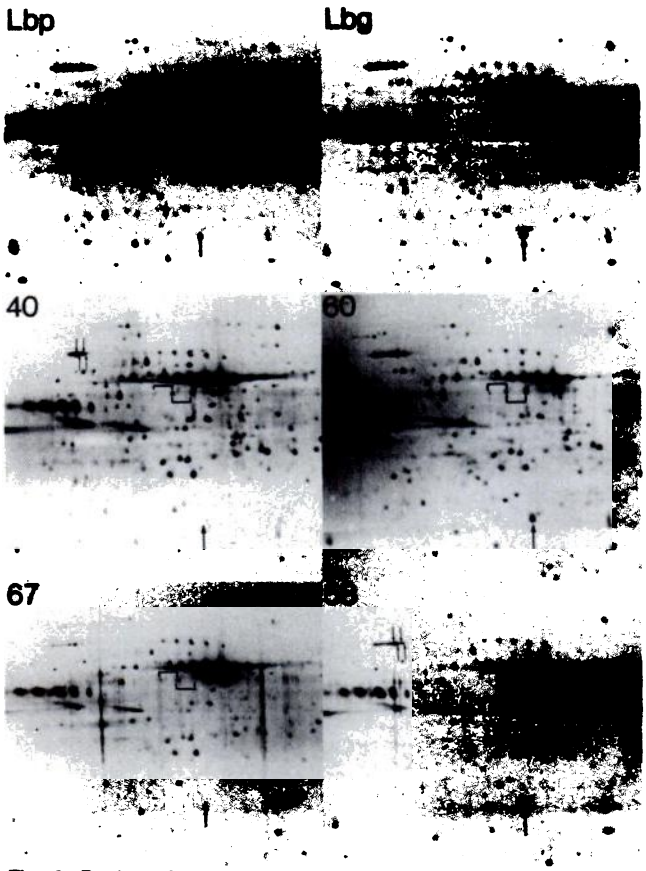

Fig. 6. Portion of two-dimensional electrophoretic patterns of *L. b. panamensis* (*Lbp*) and *L. b. guyanensis* (*Lbg*) reference strains and Colombian isolates displaying intermediate phenotypes

Arrows indicate selected distinctive elements in the overall comparison

References

1. Manson-Bahr PEC, Apted FIC. Cutaneous leishmaniasis of the New World. In *Mansons Tropical Diseases,* 18th ed., Bailliere, Tindall, London, 1982, pp 109–115.

2. Shaw PK, Quigg LT, Allain DS. Autochthonous cutaneous leishmaniasis in Texas. *Am J Trop Med Hyg* 25, 788–796 (1976).

3. Lainson R, Shaw JJ. Leishmaniasis of the New World: Taxonomic problems. *Br Med Bull* 28, 44–48 (1972).

4. Walton BC, Intermill RW, Hajduk ME. Differences in biological characteristics of three *Leishmania* isolates from patients with espundia. *Am J Trop Med Hyg* 26, 850–855 (1977).

5. Walton BC, Shaw JJ, Lainson R. Observations on the *in vitro* cultivation of *Leishmania braziliensis. J Parasitol* 63, 1118–1119 (1977).

6. Lainson R, Ward RD, Shaw JJ. *Leishmania* in phlebotomid sandflies: VI. Importance of hindgut development in distinguishing between parasites of the *Leishmania mexicana* and *Leishmania braziliensis* complexes. *Proc R Soc Lond* 199, 309–320 (1977).

7. Chance ML, Gardener PJ, Peters W. Biochemical taxonomy of *Leishmania.* I Observations on DNA. *Am Trop Med Parasitol* 72, 533–542 (1974).

8. Chance ML, Gardener PJ, Peters W. Biochemical taxonomy of *Leishmania* as an ecologic tool. Ecologie des Leishmanioses. *Colloq Int CNRS* 239, 53–62 (1977).

9. Barker DC, Arnot DE. Biochemical identifications of cutaneous leishmaniasis by analysis of kinetoplast DNA. I Ultrastructural and bouyant density analysis. *Mol Biochem Parasit* 3, 33–46.

10. Decker-Jackson JE, Schrot JR, Levin GV. Identification of *Leishmania* spp. by radiorespirometry. *J Protozool* 24, 463–470 (1977).

11. Schnur LF, Zuckerman A, Greenblatt CL. Leishmanial serotypes as distinguished by gel diffusion of factors excreted *in vitro* and *in vivo. Isr J Med Sci* 8, 932–942 (1972).

12. Wirth D, McMahon-Pratt D. Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. *Proc Natl Acad Sci USA* 79, 6999–7003 (1982).

13. Baker DC, Butcher J. The use of DNA probes in the identification of leishmanias: Discrimination between isolates of the *Leishmania mexicana* and *L. braziliensis* complexes. *Trans R Soc Trop Med Hyg* 77, 285–297 (1983).

14. Miles MA, Povoa MM, DeSouza AA, et al. Some methods for the enzyme characterizations of Latin American *Leishmania* with particular reference to *Leishmania mexicana amazonensis* and subspecies of *Leishmania hertigi. Trans R Soc Trop Med Hyg* 74, 244–252 (1980).

15. Miles MA, Lainson R, Shaw JJ, et al. Leishmaniasis in Brazil: XV. Biochemical distinction of *Leishmania mexicana amazonensis, L. braziliensis,* and *L. braziliensis guyanensis*—aetiological agents of cutaneous leishmaniasis in the Amazon Basin of Brazil. *Trans R Soc Trop Med Hyg* 75, 524–529 (1981).

16. Lainson R, Shaw JJ, Ready PD, et al. *Leishmania* in Brazil: XVI. Isolation and identification of *Leishmania* species from sandflies, wild mammals and man in North Pará state, with particular reference to *L. braziliensis guyanensis* causative agent of "pianbois." *Ibid.,* pp 530–536.

17. McMahon-Pratt D, David JR. Monoclonal antibodies that distinguish New World species of *Leishmania. Nature (London)* 291, 581–583 (1981).

18. McMahon-Pratt D, Bennet E, David JR. Monoclonal antibodies that distinguish species of *Leishmania braziliensis. J Immunol* 129, 926–927 (1982).

19. *Biochemical Characterization of Leishmania.* ML Chance, BC Walton, Eds., UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland, 1981.

20. Anderson NL, Giometti CS, Gemmell MA, et al. A two-dimensional electrophoretic analysis of the heat-shock-induced proteins of human cells. *Clin Chem* 28, 1084–1092 (1982).

21. Anderson NG, Anderson NL. Analytical technique for cell fractions. XXI Two-dimensional analysis of serum and tissue proteins: Multiple isoelectric focusing. *Anal Biochem* 85, 331–340 (1978).

22. Anderson NL, Anderson NG. Analytical technique for cell fractions. XXII Two-dimensional analysis of serum and tissue proteins: Multiple-gradient slab electrophoresis. *Ibid.,* pp 341–354.

23. Guevara J. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels. *Electrophoresis* 3, 197–205 (1982).