BIOCHEMICAL IDENTIFICATION OF *LEISHMANIA* SPP BY CELLULOSE ACETATE ELECTROPHORESIS

by

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ABSTRACT

BIOCHEMICAL IDENTIFICATION OF LEISHMANIA SPP.

BY CELLULOSE ACETATE ELECTROPHORESIS

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Master of Science

Youngstown State University, 1984

Cellulose acetate electrophoresis has been proven to be an accurate, simple and rapid method for biochemical (genetic) identification of Leishmania isolates to the subspecific level. Some enzyme systems (glucose phosphate isomerase (GPI), glutathione reductase NADP (GSR_1), isocitrate dehydrogenase (ICD), malate dehydrogenase (MDH), malic enzyme (ME), mannose phosphate isomerase (MPI) and phosphogluconate dehydrogenase (6-PGDH) were modified so that the buffer and stain components can be packaged which further facilitates the identification process. Two new enzyme systems, alkaline phosphatase (ALP), and glutathione reductase NAD (GSR_2), were introduced. ALP was of limited value while GSR_2 could be added to the preceding seven since it does not require a pH meter and yields good bands with good separation between subspecies. Eighty-six isolates from Walter Reed Army Institute of Research and two from the National Institute of Health in Columbia were identified by this technique using twenty-five enzyme systems. Since Leishmania species can be expected to be 21% polymorhpic, isolates with similarities greater than 75% between each other were considered to be of the same subspecies. The results obtained were as follows: 35

isolates were found to be Leishmania braziliensis panamensis, 9 L. b. braziliensis, 5 L. mexicana mexicana, 10 L. m. venezuelensis, 6 L. donovani, 7 L. major, 6 L. tropica, 1 L. m. aristedesi, 1 L. m. pifanoi, 4 Leishmania sp., 3 still listed as unknowns but could be Leishmania sp. and 1 Herpetomonas. The causative agent of the diffuse cutaneous leishmaniasis (DCL) was found to belong to a specific subspecies of the L. mexicana complex, which could be L. m. venezuelensis. It was 49% similar to L. m. mexicana, had its own enzyme "profile" and was widely spread geographically. It can be separated from the other Leishmania types by the study of only two enzyme systems, GPI and MPI. L. major and L. tropics types were found to have distinct enzyme "profiles"; they were less than 30% identical. In addition, three samples from lizards, L. adleri, L. agamae and L. tarentolae, as well as two isolates from guinea pigs, L. m. enriettii, were run for most of the twenty-seven enzyme systems. The lizard subspecies had low similarities (less than 40%) with any of the major groups, while the L. m. enriettii was 35% similar to L. m. mexicana. Finally, with the increaed number of isolates run, more subspecies were found from the same geographical area so that identification based on the country of origin has shown to be unreliable.

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CHAPTER I

INTRODUCTION

Leishmaniasis was considered in 1976 by the World Health Organization (WHO) as being among the six major tropical diseases in the world. From the standpoint of human sufferings and economics it is probably only second to malaria in importance among the protozoal diseases; while the treatment and control are often much more difficult (Lainson 1980). Although the incidence of human leishmaniasis is generally believed to be regressing, it is estimated that a "few million" people are still affected. Endemic foci of leishmaniasis are scattered over wide areas of the globe, mainly tropical and neotropical, such as Asia, Africa, the Mediterranean Basin and Central and South America.

The genus Leishmania has two distinct morphological stages: an intracellular amastigote in the mammalian host and an extracellular flagellated promastigote in the insect vector. The sandflies Phlebotomus spp. and Lutzomya sp. are the major vectors and intermediate hosts, while the small mammals are reservoir hosts and man in most cases an accidental one (Lainson, 1980). Although these two stages differ considerably in structure and morphology, few if any interspecific differences have been reported (Al-Taqi and Evans 1978, Kassner 1968, Lumsden 1974). No morphological differences can be observed between the intracellular stages of Leishmania spp. (with the exception of the distinctly larger L. hertigi). Also, the flagellates in culture are similar or show minor or inconsistent differences (Adler 1964). Leishmania —— .provides an example of how slight differences between closely related pathogens can

be of considerable epidemiological significance (Adler 1964). Three clinical types of leishmaniasis are associated with specific organisms: visceral leishmaniasis (L. donovani), mucocutaneous leishmaniasis (L. braziliensis), and cutaneous leishmaniasis (L. tropica and L. mexicana) (Marsden 1979). An interesting form of the disease is the diffuse cutaneous leishmaniasis (DCL) which has been described in Africa and South America. It starts as a single lesion which spreads slowly, until it may cover the whole body. The DCL, a generalized disease of the skin, may be due to an immunological defect of the host (Convit et al 1972) and seems to be limited in the Americas to infections with subspecies of &. mexicana.

Infections of Leishmania in man range from a single, mild, self-healing, localized ulceration to a widespread cutaneous and destructive mucocutaneous involvement or to a severe fatal visceral form (Price and Silver 1977). The degree of clinical variation is probably dependent both on host reaction to the parasite and on the particular infecting strain of Leishmania (Price and Silver 1977, Chance 1979). The definition of subspecies using only clinical and epidemiological criteria is very unsatisfactory and has led to many confusions and misconceptions. Indeed, the initial cutaneous lesion, which is similar in all the clinical entities, will either heal spontaneously or spread further (DCL, mucocutaneous, visceral).

Identification of Leishmania spp. is a world health priority and of importance to research on the taxonomy of this genus as well as to studies on the epidemiology, diagnosis and treatment of leishmaniasis (Gardener 1974, Chance 1979, Miles et al 1979). Furthermore, the long term prospect of controlling leishmaniasis by vaccination will only be

achieved by having the exact knowledge of the identity of the parasite from which antigen is prepared (Chance 1979). The increase in travel to endemic areas, the presence of military personnel in these countries, and the travel of possibly infected natives to countries where the disease does not exist has made it a difficult task for the physicians and clinicians in the identification of the disease. Clearly, it is beyond the scope of most clinicians to bring diagnosis down to the subspecific nature of the Leishmania concerned, particularly when dealing with American cutaneous leishmaniasis. However, attempts should be made to have the organisms isolated and passed on to experts for more accurate identification (Lainson 1980).

Despite the morphological similarities among subspecies of Leishmania, the biochemical and genetic differences present between them make the latter good tools in identification (Chance et al 1978). Early identification studies (Chance et al 1978, Chance 1979, Marsden 1979) used comparative buoyant densities of nuclear and kinetoplast DNA; this method proved to be of particular value at the generic and specific levels but of limited value at the lower taxa. It also requires a costly apparatus and is limited to use by a few investigators. Radiorespirometry and other increasingly sophisticated methods may indicate differences so fine as to render their significance difficult (Lainson 1980).

Characterization leading to identification of Leishmania subspecies by enzyme electrophoresis has been brought to a high level of efficiency (Lainson 1980). Populations belonging to different species almost invariably show considerably more genetic differences than do conspecific populations (Avise 1975). Since cell proteins may be gradually modified by genetic mutation, it is possible to assess the

relatedness of organisms by comparing the structure of their homologous proteins (Gibson et al 1980). High levels of genetic similarities were found between conspecific populations with mostly identical allelic (allozyme) content in 85% or more of their loci (Ayala 1975, Ayala et al 1974, Kreutzer et al 1982). Enzyme electrophoresis is the migration of polypeptides through an electric field at different rates usually according to their net charge (Avise 1975). Thus, proteins which migrate different distances usually differ by at least one amino acid substitution; the colinearity of amino acid sequence and nucleotide sequence in the DNA implies that the proteins were encoded by segments of DNA differing in at least one base pair. Isozymes are proteins catalysing the same reaction (use the same substrate) but which differ in their physical and chemical properties and so differ in electrophoretic mobility (Kassner 1968). However, allozymes which are different electrophoretic form of the protein, caused by allelic difference, may be caused by a different amino acid sequence in one or more of the polypeptide chains that compose the protein and so might migrate different distances from one of the poles (Strickberger 1968). Thus electrophoretic mobility of proteins provides indirect information about DNA (Avise 1975, Chance 1979, Tibayrenc et al 1979). Since the substitution of an amino acid for another with an equal change will not be detected, Harris and Hopkinson (1976) estimated that only a third of possible amino acid substitutions would result in alteration of electrophoretic mobility. Nevertheless, the more enzymes that are electrophoretically identical between two samples, the greater is the likelihood that the two enzymes samples are from related organisms; the greater the number of identities, the closer is the relationship (Gibson 1980, Tibayrenc et al 1979).

Previous studies of Leishmania sp. identification by Al-Taqi and Evans (1978), Ashford (1976), Chance et al (1978), Chance (1980), Gardener (1972), Godfrey (1978) and Miles et al (1977) used gel electrophoresis on ten enzyme systems, but recently a simplified system, cellulose acetate electrophoresis (CAE), has been used by Kreutzer and Christensen (1980), and Kreutzer et al (1982).

A recent paper Kreutzer et al (1982) reported electrophoretic techniques on cellulose acetate for twenty-five enzymes (possibly 29 loci); 21% of the enzymes were polymorphic and of lesser value in identification, while others were monomorphic and of taxonomic significance. The authors proposed that because of the naturally occurring polymorphism, isolates with enzymes "profiles" greater than 75% identical are of the same subspecies and isolates which are significantly less than that are samples from different subspecies. Data from this study suggest that among the isolates tested there were five major complexes of Leishmania: L. braziliensis, L. mexicana, L. donovani, 4. tropica and L. hertigi.

Taxonomic "profiles" must be based on multiple enzyme systems and on data from many isolates representing entire geographical distribution of the group (Kreutzer et al 1982). The subspecies were named after the specific area of their occurrence, such as L. b. panamensis from-panama and Costa Rica, L. b. braziliensis from Brazil, whereas, L. m. mexicana from Mexico, Guatemala, and Belize, but only a few isolates from'these and other areas were identified with CAE. In the following study a larger number of isolates was available and as will be seen more subspecies are being found in each country with some of the subspecies being very

widely spread. Also, in the following paper the causative agent of the DCL is identified by CAE (Kreutzer et al., in publication).

Finally, in previous studies based on data from small numbers of isolates, CAE has proven useful, rapid, and accurate in the identification of Leishmania subspecies. Most of the infections occur in field conditions with no sophisticated material available, so the present research, on 93 different isolates (88 of them from Walter Reed Army Institute of Research (WRAIR)). expands and modifies the earlier work to increase sensitivity, to determine more accurately polymorphism and to simplify an already simple technique to suit the need of the field diagnosis.

CHAPTER II

MATERIALS AND METHODS

Leishmania1 strains

The 93 Leishmania isolates used were mostly from WRAIR. They were identified previously by classical methods and techniques. Details on their identification, the disease caused and the countries from where they were isolated are listed in Table 1. The identification listed, is the final one to the subspecific level using CAE, while the unidentified isolates are listed as Leishmania sp. or unknowns. The controls used for comparisons have been previously identified by CAE by Kreutzer et al (1983) and are listed in Table 2.

Cultivation

Some of the isolates were received as buttons of packed cells ready to be lysed, while others were cultivated in the laboratory following the procedures of Hendricks et al (1978) and Childs et al (1978). Two media were used, one of Schneider's Drosophila medium supplemented with 20% (v/v) Fetal Bovine Serum (FBS) and .1 ml gentamycin, the other of NNN (Novy-MacNeal-Nicolle) biphasic agar-rabbit medium with a 20 ml overlay of the preceeding Schneider's-FBS-Gentamycin medium. The cultures were kept in an incubator at 24± 1°C until they produced about 3×10^9 promastigote cells before being centrifuged at 2800xg (15°C) for 10 min., washed once with 1 ml of normal saline and preserved in the cryogenic tank in liquid nitrogen at -196°C.

The button of packed cells is overlayed with an appropriate amount of buffer (14 parts distilled water: 1 part 0.1M Trizma base (Tris)/ 0.1M Maleic acid/ 0.01M EDTA (Na_2)/ 0.01M Magnesium chloride

LEISHMANIA ISOLATES

CAE Species or Species/Type	WRAIR No.	Other Designations	Host	Disease	Country
L. braziliensis panamensis	003	Husbands	Human	NWC	Panama
L. braziliensis panamensis	004	Marlow	Human	NWC	Panama
L. braziliensis panamensis	111C	Salgado	Human	V	Belize
L. braziliensis panamensis	111N	Salgado	Human	V	Belize
L. braziliensis panamensis	132	Tush	Human	NWC	Panama
L. braziliensis panamensis	154	Hernandez	Human	NWC	Panama
L. braziliensis panamensis	176	Watterson	Human	NWC	Panama
L. braziliensis panamensis	179	Legoas	Human	NWC	Panama
L. braziliensis panamensis	211	Fizer	Human	NWC	Panama
L. braziliensis panamensis	232	Barstow	Hamster	NWC	Panama
L. braziliensis panamensis	241	Martin	Human	NWC	Panama
L. braziliensis panamensis	246	Boynton	Human	NWC	Panama
L. braziliensis panamensis	282	Morrison	Human	NWC	Panama
L. braziliensis panamensis	322	Kingsley	Human	NWC	Panama
L. braziliensis panamensis	315	Blownt	Human	NWC	Canal Zone
L. braziliensis panamnesis	360	Clonts	Human	NWC	Panama
L. braziliensis panamensis	380	Coffey	Human	NWC	Panama
L. braziliensis panamensis	390	Ward	Human	NWC	Brazil
L. braziliensis panamensis	442	Bennet	Human	NWC	Panama
L. braziliensis panamensis	446	Burgos	Human	NWC	Panama
L. braziliensis panamensis	470	Belisle	Human	NWC	Panama
L. braziliensis panamensis	475	Varsel	Human	NWC	Panama
L. braziliensis panamensis	486	Reinertsen	Human	NWC	Panama
L. braziliensis panamensis	487	Tyus	Human	NWC	Panama
L. braziliensis panamensis	491	Reinertsen	Human	NWC	Panama
L. braziliensis panamensis	492	Reinertsen	Human	NWC	Panama
L. braziliensis panamensis	493	Reinertsen	Human	NWC	Panama
L. braziliensis panamensis	505	Reyes	Human	NWC	Panama
L. braziliensis panamensis	525	DominquesL1	Human	NWC	Panama
L. braziliensis panamensis	526	DominquesL2	Human	NWC	Panama

TABLE 1 (CONTINUED)

LEISHMANIA ISOLATES

CAE Species or Species/Type	WRAIR No.	Other Designations	Host	Disease *	Country
L. braziliensis panamensis	530	Schoonmaker	Human	NWC	Panama
L. braziliensis panamensis	535	Tyus	Hamster	NWC	Panama
L. braziliensis panamensis	539	Carillo	Human	NWC	Panama
L. braziliensis panamensis	556	Evans	Human	NWC	Panama
L. braziliensis panamensis	578	Castro-placenta		NWC	Panama
L. braziliensis braziliensis	359	Courtwright	Human	NWC	Panama
L. braziliensis braziliensis	410	Askew	Human	NWC	Panama
L. braziliensis braziliensis	508	Barbosa	Human	NWC	Brazil
L. braziliensis braziliensis	540	Bel-10	Human	NWC	Belize
L. braziliensis braziliensis	541	Bel-14	Human	NWC	Belize
L. braziliensis braziliensis	542	Bel-15	Human	NWC	Belize
L. braziliensis braziliensis	543	Bel-17(A)	Human	NWC	Belize
L. braziliensis braziliensis	545	Bel-25	Human	NWC	Belize
L. braziliensis braziliensis	557	Bel-16	Human	NWC	Belize
L. mexicana mexicana	-	019	-----	---	Columbia
L. mexicana mexicana	347	Nyctomys sumichraski	-----	---	Belize
L. mexicana mexicana	524	Alderman	Human	NWC	Panama
L. mexicana mexicana	531	Castro	Human	NWC	Panama
L. mexicana mexicana	585	Jone	Mouse	C	------
L. mexicana venezuelensis	348	------	Human	NWC	Costra Rica
L. mexicana venezuelensis	381	Peters	Human	NWC	Panama
L. mexicana venezuelensis	411	------	Human	NWC	Texas
L. mexicana venezuelensis	447	------	Human	NWC	Dom. Rep.
L. mexicana venezuelensis	453	------	Human	NWC	Dom. Rep.
L. mexicana venezuelensis	457	Lump 543, LV96	Human	NWC	Venezuela
L. mexicana venezuelensis	468	------	Human	NWC	Dom. Rep.
L. mexicana venezuelensis	527	Gladys, LV91, DCL	Human	DCL	Venezula
L. mexicana venezuelensis	548	Bel-18	Human	NWC	Belize
L. mexicana venezuelensis	549	Bel-26	Human	NWC	Belize
L. donovanf chagasi	285	Wade	Human	NWC	Panama

TABLE 1 (CONTINUED)

LEISHMANIA ISOLATES

CAE Species or Species/Type	WRAIR No.	Other Designations	Host	Disease*	Country
L. donovani chagasi	317	Natuel	Human	NWC	Panama
L. donovani chagasi	341	Furr	Human	NWC	Panama
L. donovani chagasi	520	FRACISCO	Human	NWC	Brazil
L. donovani	555	Hanson	Hamster	OW	Khartoom
L. donovani	311	David Wambua	Human	OWV	Kenya
L. major	547	LRC L137	Human	OWC	Kenya
L. major	551	NLB 095	Rodent	OWC	Kenya
L. major	552	NLB 144	Sandfly	OWC	Kenya
L. major	558	Beach	Human	OWC	Kenya
L. major	559	Beach	Human	OWC	Kenya
L. major	586	WR 508	-----	NWC	-----
L. major	587	WR 470	-----	NWC	-----
L. tropica	---	031	-----	---	Columbia
L. tropica	218	Rece	Human	NWC	Canal Zone
L. tropica	581	Ackerman	Human	C	NIH
L. tropica	582		Human	C	NIH
L. tropica	583	Friedlin	Human	C	NIH
L. tropica	584	Niazy	Human	C	NIH
L. mexicana aristedesi	481	Oryzomys	-----	NWC	Panama
L. mexicana pifanoi	528	NR	Human	DCL	Venezula
L. enrietti	516	Weinstein	Guinea pig	NWC	Unknown
L. enrietti	529	Weinstein	Guinea pig	NWC	-------
L. adleri	---	---------	Lizard	---	-------
L. agamae	---	---------	Lizard	---	-------
L. tarentolae	---	---------	Lizard	---	-------
Leishmania sp.	316	Wilcoxen	Human	NWC	Panama
Leishmania sp.	544	Bel-21(C)	Human	NWC	Belize
Leishmania sp.	553	---------	Human	NWC	Ecuador
Leishmania sp.	554	---------	Human	NWC	Ecuador

TABLE 1 (CONTINUED)

LEISHMANIA ISOLATES

CAE Species or Species/Type	WRAIR No.	Other Designations	Host	Disease*	Country
Herpetomonas	523	Zelus leucogrammus	----	---	Brazil
Unknown	177	Llewellyn	Human	NWC	Brazil
Unknown	440	Joseph Kiptoo	Human	V	Kenya
Unknown	495	----------	Human	V	Kenya

*
NWC, new world cutaneous leishmaniasis; V, visceral leishmaniasis; DCL, diffuse cutaneous leishmaniasis; C, cutaneous leishmaniasis; OWV, old world visceral leishmaniasis; OWC, old world cutaneous leishmaniasis.

TABLE 2

LEISHMANIA ISOLATES USED AS CONTROL

CAE Species or Species/Type	WRAIR No.	Other Designations	Host	Disease*	Country
L. braziliensis panamensis	008	Murray	Human	NWC	Canal Zone
L. braziliensis panamensis	111	Salgado	Human	NWV	Belize
L. braziliensis panamensis	128	Murray, K.	Hamster	NWC	Canal Zone
L. braziliensis braziliensis	294	Jurandi	Human	NWC	Brazil
L. mexicana mexicana	225	Huggins	Human	NWC	Panama
L. mexicana venezuelensis	140	-	Human	DCL	Peru
L. mexicana venezuelensis	183	-	Hamster	NWC	Belize
L. mexicana venezuelensis	338	Isabella, same as NIH/Isabel	Human	DCL	Dom. Rep.
L. donovani	130	Khartoum	Human	-	Sudan
L. donovani	116	ATCC 30881 (Herrera)	Human	-	Honduras
L. donovani	425	NL BOO8, Mutiso	Human	-	Kenya
L. major	308	LRC-L207, old Israeli vaccine strain	Human	OWC	Israel

*NWC, New world cutaneous leishmaniasis; NWV, New world visceral leishmaniasis; DCL, diffuse cutaneous leishmaniasis; OWC, Old world leishmaniasis

($MgCl_2$), and the pH is adjusted to 7.4 with 40% NaOH), ~ .25 ml buffer for a button of cells 4–5 mm in diameter; more or less buffer for a larger or smaller pellet. Then the cells are suspended in the buffer with a vortex, and lysed by three freeze and thaw cycles. Finally, the solution is centrifuged at 2800xg (15°C) for 10 min., the supernatant removed and preserved at -196°C in liquid nitrogen until being used for electrophoresis.

Conditions for electrophoresis

The enzymes used for characterization are listed in Table 3 along with their abbreviation, their identification number and their number of loci. The enzyme systems are taken from Kreutzer and Christensen (1980) and Kreutzer et al (1983), with modifications in some, to simplify them for field purposes. The buffer/substrate/stain components used in electrophoresis are listed in Table 4, they include the cell buffer, membrane buffer, reaction buffer, substrate/stain components, the voltage and time required and the number of applications needed normally for each enzyme system. The enzyme systems which were modified in this study are: Glucose phosphate isomerase (GPI), Glutathione reductase$_1$ (GSR_1), Isocitrate dehydrogenase (ICD), Malic enzyme (ME), Malate dehydrogenase (MDH), Mannose phosphate isomerase (MPI) and Phosphogluconate dehydrogenase (6–PGDH). Their components were calculated so that upon addition of distilled water the required pH and mobility are obtained.

Substrate/stain plates were prepared following the instructions in Table 4. Two portions each of 25 ml reaction buffer were placed one over the substratefstain components, the other over .5g of 20% noble agar, the latter was then brought to a boil and cooled to about 50°C before pouring it over the 25 ml of the substratefstain portion. Then this

TABLE 3

ENZYMES TESTED IN THIS STUDY[a]

Enzyme	Enzyme Commission Number	Enzyme Abbreviation	Rating[b]	Number of Loci[c]
Oxidoreductases				
Lactate dehydrogenase	1.1.1.27	LDH	2-3	1-2
Malate dehydrogenase	1.1.1.37	MDH	1	1-2
Malic enzyme	1.1.1.40	ME	1-2	1
Isocitrate dehydrogenase	1.1.1.42	ICD	2	1-2
Phosphogluconate dehydrogenase	1.1.1.44	6-PGDH	1	1
Glucose-6-phosphate dehydrogenase	1.1.1.49	G-6-PDH	1	1-2
Glyceraldehyde-phosphate dehydrogenase	1.2.1.12	GAPDH, GAPDH$_2$	2	2 or more
Glutathione reductase	1.6.4.2	GSR$_1$	1-2	1-1
Glutathione reductase(2)	1.6.4.2	GSR$_2$	1	
Transferases				
Glutamate-oxaloacetate transaminase	2.6.1.1	GOT & ASAT	1-2	1
Glutamate-pyruvate transaminase	2.6.1.2	ALAT	1	1
Hexokinase	2.7.1.1	HK	2	1-3
6-Phosphofructokinase	2.7.1.11	FK	2-3	1-2
Adenylate kinase	2.7.4.3	AK	1-2	1-2
Guanylate kinase	a.7.4.8	GUK	4	1-2
phosphoglucomutase	2.7.5.1	PGM	1-2	1-2
Hydrolases				
Esterases	3.1.1.1	EST	2-3	1-2
Alkaline phosphatase	3.1.3.1	ALP	4	1
Acid phosphatase	3.1.3.2	ACP	1	1-3
Peptidases	3.4.11.13	PEP	1	1
Peptidases D	3.4.13.9	PEPD	1	1
Lyases				
Aldolase	4.1.2.13	ALD	4	1-2
Fumerate hydratase	4.2.1.2	FUM	2	1-2
Isomerases				
Mannose phosphate isomerase	5.3.1.8	MPI	1	1
Glucose phosphate isomerase	5.3.1.9	GPI	1	1

[a]Commission on Biochemical Nomenclature, 1973.

[b]The ratings indicate facility of operation. Enzyme systems which produce distinct, rapidly appearing bands from a single application of the isolate are rated 1.

[c]Enzymes which regularly produce more than one band for most isolates are possibly multi-isozyme systems; however, no substantiating data is available.

TABLE 4

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Membrane Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	SUBSTRATE STAIN COMPONENTS*
ACP[a,c]	1	1:14	160	15	A	75 mg Naphthol as phosphoric acid; 50 mg Fast Garnet GBC.
AK[c]	2	1:10	160	15	B	30 mg Adenosine 5-Diphosphate ADP(Na_2 ; 120 mg Magnesium Chloride ($MgCl_2$) $6H_2O$; 150 mg α-D(+) glucose; 15 mg β-Nicotinamide Adenine Dinucleotide Phosphate (β-TPN), Na salt; 15 mg MTT Tetrazolium (MTT); 10 mg Phenazine methosulfate (PMS); 15 units Glucose-6-phosphate dehydrogenase; 30 units Hexokinase (0.1 ml).
ALAT[c]	3	1:9	160	10	E	30 mg α-Ketoglutaric acid (Na salt); 50 mg L-alanine, 30 units (~15$\mu\ell$) Lactic Dehydrogenase (beef heart in phosphate buffer pH 7.5); 15 mg β-Nicotinamide Adenine Dinucleotide, (β-NAD) (Na_2 Salt).
ALD[a]	3	1:9	160	15	F	250 mg Fructose-1, 6-Diphosphate (Na_4 salt); 15 mg MTT; 15 mg β-Nicotinamide Adenine Dinucleotide (β-DPN); 10 mg PMS; 300 units (.3 ml) Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle $(NH_4)_2\ SO_4$ solution, pH 7.5); 60 mg Sodium Arsenate.
ALP[a]	1	1:14	200	10	G	75 mg β-Naphthyl Phosphate; 100 mg Magnesium Sulfate ($MgSO_4$); 30 mg Fast Blue RR.

TABLE 4 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Membrane Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	SUBSTRATE/STAIN COMPONENTS*
ASAT[c]	1	1:14	180	15	E	30 mg α-Ketoglutaric acid (Na salt); 70 mg L-Aspartic acid, readjust pH to 7.4; 100 units (.03 ml) Malic dehydrogenase (Porcine heart in Phosphate buffer .02 ml pH 7.5); 15 mg β-NADH (Na_2 salt).
EST[b]	4	1:14	180	15	H	25 mg 4-methylumbelliferyl acetate (dissolve in 5 ml 100% Acetone).
FK[a,c]	3	1:5	160	15	I	200 mg β-D-(-)Fructose; 15 mg MTT; 15 mg β-TPN (Na salt); 10 PMS; 40 mg Adenosine Triphosphate (ATP); 20 mg $MgCl_2$; 50 units (.05 ml) Phosphoglucose isomerase.
FUM[a,c]	2	1:7	180	15	C	.5 g Fumaric acid (K salt), readjust to pH 7.0; add 15 mg β-DPN (Na_2 salt); 15 mg MTT; 10 mg PMS; 200 units (30 μl) Malic dehydrogenase.
$GAPDH_1$[a]	2	1:14	300	15	D	80 mg Fructose-1, 6-diphosphate (Na_3 salt, 7 H_2O), dissolve in 2 ml reaction buffer; Add .05 ml Aldolase and incubate at 37°C for 1 hour; Then add 30 mg β-DPN (Na_2 salt); 50 mg Sodium Arsenate; 50 mg sodium pyruvate; 5 mg MTT; 2.5 mg PMS.
$GAPDH_2$[a]	2	1:5	220	10	D	100 mg Fructose-1, 6-diphosphate (Na salt, 7 H_2O); dissolve in 2 ml reaction buffer; add 1 ml Aldolase and incubate at 37°C for 1 hour; then add 20 mg β-DPN (Na_2 salt), 15 mg MTT; 10 mg PMS; 100 mg Sodium Arsenate; 50 mg Sodium pyruvate.

TABLE 4 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Membrane Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	SUBSTRATE/STAIN COMPONENTS*
GOT	2	1:15	180	15	I	Substrate: 100 mg L-Aspartic acid; 75 mg α-Ketoglutaric acid, reapjust to pH 8.0; add 10 mg Pyridoxal-5-phosphate; pour this mixture with agar by itself in plates. Stain: 75 mg Fast blue BB.***
GPI	5	1:14	180	15	J	20 mg Fructose-6-phosphate; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 60 mg Ethylenediaminetetraacetic acid (EDTA); 25 units (1 flake) Glucose-6-phosphate dehydrogenase (Bakers yeast).
G-6-PDH[a]	3	1:14	160	15	F	75 mg D-Glucose-6-phosphate; 15 mg MTT; 15 mg β-TPN (Na salt), 10 mg PMS
GSR_1[a]	3	1:9	180	12	K	30 mg Oxidized Glutathione; 5 mg β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced form (NADPH), < 1 mg ea, 6-Dichlorophenol-indophenol; 15 mg MTT.
GSR_2[a]	3	1:9	200	15	K	30 mg Oxidized Glutathione; 5 mg β-NADH (Na_2 salt); <1 mg 2, 6-Dichlorophenol-indophenol; 15 mg MTT.
GUK[b]	6	1:10	250	15	L	30 mg Adenosine 5-Triphosphate (Na_2 salt from equine muscle); 50 mg Guanosine-5-monophosphoric acid (GMP), Na_2 salt; 15 mg Phosphoenolpyruvate (K salt); 15 mg β-DPN (Na_2 salt), Na_2 salt; 60 mg $MgCl_2$; 150 mg Potassium chloride (KCl); 30 mg $CaCl_2$; 280 units (.06 ml) Lactic dehydrogenase (from bovine heart); 20 units (.03 ml) Pyruvate Kinase (from rabbit muscle $(NH_4)_2 SO_4$ solution pH 6.0).

TABLE 4 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Membrane Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	SUBSTRATE/STAIN COMPONENTS*
HK	7	1:5	160	15	F	50 mg α-D(+)-Glucose; 20 units (1 flake) Glucose-6-phosphate dehydrogenase; 20 mg $MgCl_2$; 15 mg MTT; 15 mg β-TPN (Na salt) (ATP).
ICD[a]	3	1:14	180	15	N	100 mg DL-Isocitric acid (Na_3), readjust to pH 8.0; add 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS.
LDH[a]	3	1:14	160	15	I	3 ml DL-Lactic acid (Na salt); 15 mg β-DPN (Na_2 salt); 15 mg MTT; 10 mg PMS.
MDH[b]	8	1:9	200	15	E	15 mg oxalacetic acetic; 15 mg β-NADH (Na_2 salt).
ME	5	1:14	180	15	M	270 mg DL-Malic acid; 604 mg Trizma base (Tris); 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 mg Manganese chloride ($MnCl_2$).
MPI	9	1:5	160	15	O	15 mg Mannose-6-phosphate; 10 mg β-TPN (Na_2 salt); 10 mg MTT; 5 mg PMS; 1 mg Glucose phosphate isomerase; 15 units (1 flake) Glucose-6-phosphate dehydrogenase.
6-PGDH[a]	3	1:14	180	15	J	15 mg 6-Phosphogluconic acid (Na_3 salt); 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 60 mg EDTA (Na_2 salt).

TABLE 4 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Membrane Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	SUBSTRATE/STAIN COMPONENTS*
PGM	2	1:14	180	15	J	200 mg α-D-Glucose-1-phosphate; 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 units (1 flake) Glucose-6-phosphate dehydrogenase (Bakers yeast); 60 mg EDTA (Na_2 salt); 1.2 mg α-D-glucose-1, 6-diphosphate.
PEP[b]	10	1:14	160	10	P	20 mg L-leucyl-L-Alanine; 1.5 mg L-Amino acid oxidase (Crotalus adamanteus venom); 2 mg Peroxidase 20 mg Manganese chloride ($MnCl_2$); 15 mg 3-Amino-9-ethylcarbazole.
PEPD[b]	10	1:14	160	10	P	20 mg L-Leucyl-L-Proline (hydrochloride); 1.5 mg L-Amino acid oxidase (Crotalus adamateus venom); 2 mg Peroxidase; 20 mg Manganese Chloride; 15 mg 3-Amino-9-ethylcarbazole.

Cell buffers:

1. .15 M Citric acid (Na_3)/.24 M Sodium phosphate monobasic (NaH_2PO_4), adjust to pH 6.3 with 40% Sodium Hydroxide NaOH.
2. .1 M Trizma base (Tris)/.1 M Maleic acid/.01 M Ethylenediamine tetra-aceticacid (EDTA) (Na_2), 1.01 M Magnesium Cloride ($MgC\ell_2$), adjust to pH 7.4 with 40% NaOH.
3. .7 M Sodium phosphate monobasic (NaH_2PO_4)/.13 M Sodium phosphate dibasic (Na_2HPO_4), pH 7.0.
4. .1 M Tris/.01 EDTA/.36 M Boric acid crystals, pH 7.2.
5. .1 M Tris/.05 M Maleic Acid/.01 M EDTA/.11 M Sodium phosphate dibasic (Na_2HPO_4) (16g/1ℓ), pH 7.4.
6. .1 M Tris/.1 M Maleic anhydride/.01 M $MgC\ell_2$, adjust to pH 7.4 with 40% NaOH.
7. .233 M Tris/.065 M Citric acid (monohydrate), adjust to pH 7.0 with 50% Hydrochloric acid ($HC\ell$).
8. .29 M Tris/.09 M Citric acid monohydrate, pH 7.0.
9. .05 M Tris/.05 M Sodium phosphate monobasic (NaH_2PO_4), pH 7.5.
10. .1 M Tris/.1 M Sodium phosphate monobasic (NaH_2PO_4), adjust to pH 7.4.

Reaction buffers:

A. .002 M Citric acid anhydrous(3.84g/1ℓ)/0.08 M Sodium citrate (23.53g/1ℓ).
B. 0.5 M Trizma base (Tris), adjust to pH 8.0 with 50% Hydrochloric acid ($HC\ell$).
C. 0.038 M Sodium phosphate monobasic (NaH_2PO_4)/0.015 M Sodium phosphate dibasic (Na_2HPO_4), pH 7.0.
D. 0.05 M Tris, adjust to pH 7.5 with 50% $HC\ell$.
E. 0.018 M Sodium phosphate monobasic (NaH_2PO_4)/0.082 M Sodium phosphate dibasic (Na_2HPO_4), pH 7.5.
F. 0.06 M Tris, adjust to pH 8.0 with 50% $HC\ell$.
G. 0.06 M Boric acid, adjust to pH 8.3 with 40% Sodium hydroxide(NaOH.
H. 0.074 M Sodium phosphate monobasic (NaH_2PO_4)/0.027 M Sodium phosphate dibasic (Na_2HPO_4), pH 6.3.
I. 0.1 M Tris, adjust to pH 8.0 with 50% $HC\ell$.
J. 0.06 M Tris (7.28g/1ℓ)/0.04 M Sodium phosphate monobasic (NaH_2PO_4) (4.72g/1ℓ), pH 8.0.
K. 0.25 M Tris (30.24g/1ℓ/0.103 M Sodium phosphate monobasic (NaH_2PO_4) (12.34g/1ℓ), pH 8.4.
L. 0.5 M Tris, adjust to pH 7.5 with 50% $HC\ell$.
M. 0.06 M Tris (7.28g/1ℓ)/0.057 M Sodium phosphate monobasic (NaH_2PO_4) (6.88g/1ℓ), pH 7.5.
N. 0.1 M Tris (12.11g/1ℓ)/0.069 M Sodium phosphate monobasic (NaH_2PO_4) (8.21g/1ℓ), pH 8.0.
O. 0.1 M Tris (12.11g/1ℓ)/0.101 M Sodium phosphate monobasic (NaH_2PO_4) (12.12g/1ℓ), pH 7.5.
0.02 M Sodium phosphate dibasic (Na_2HPO_4), adjust to pH 7.5 with 50% $HC\ell$.

Number of applications of the aliquot to the cellulose acetate plate:

Dilutions are made using 1 part buffer (0.1 M Tris/0.1 M Maleic acid/001 M EDTA/0.01 M $MgCl_2$, pH adjusted to 7.4) and 14 parts distilled water.
1:1 dilution then 1X : GPI, G-6-PDH, GSR_2, ME, PGM, PEP, PEPD.
1X : ACP, AK, ALAT, ASAT, FK, GOT, GSR, MDH, 6-PGDH.
2X : EST, ICD, LDH.
3X : ALD, FUM, GAPDH, $GAPDH_2$, HK
4X : ALP.
5X : GUK.

*To make 50 ml of stain (about 6 samples). All chemicals from Sigma.

**The membrane buffers are dilutions of 1 part cell buffer: ____ distilled water.

***The CA plates after electrophoresis are placed on substrate plates for ± 12 min. at 37°C; then blotted dry and placed on the stain plates on which the bands are monitored.

[a]These systems require cooling during electrophoresis.

[b]These systems require viewing with Ultra-Violet light for the bands to be visible.

[C]These systems have two loci.

mixture was rapidly poured into 100X15 ml petri dishes in fractions of about 7 ml in each dish. The substrate/stain must be kept from light and stored at 0-5^{o}C, until 2 min. before applying to the cellulose acetate plate after electrophoresis. The chemicals used were purchased from Sigma Chemical Company.

Electrophoresis

The cell (all the equipment for electrophoresis was manufactured by Helena Laboratories, Beaumont, Texas) is prepared by placing 100 ml portions of cell buffer in each of the side wells. A filter paper wick was placed on the cell/membrane contact surface. If cooling was needed, ice cubes were placed in the middle wells without allowing them to touch the wicks.

An aliquot of 3 µl was withdrawn from the lysate with a micro-dispenser and placed in a well of a zip zone well plate. In total, eight samples could be tested at one time making sure that no contamination occurred by good cleaning of the dispenser. Controls were always used along with new isolates. If dilution was needed, 3 µl of buffer (14 parts distilled water: 1 part 0.1M Tris/ 0.1M Maleic acid/ 0.01M EDTA/ 0.01M $MgCl_2$, and the pH is adjusted to 7.4 with 40% NaOH) was placed in the appropriate wells.

Presoaked (5 min.), Titan III cellulose acetate membranes were removed from the membrane buffers, blotted dry and placed on the super Z Aligning Base. Most of the enzymes, with the exception of three which needed a center (EST, $GAPDH_1$) or near center (PEPD) application, were applied near the bottom of the plate. About 0.5 µl of the aliquot was transferred from the well plate to the membrane with a super Z applicator. The number of applications for each enzyme is listed in Table 4.

The plates were placed enzyme side down in the cell on the filter paper wicks. Since migration is mostly anodal, the membranes were placed accordingly. The Titan power supply was set for the voltage and time desired and turned on.

After removing the plate from the cell it was blotted on each side, trimmed to fit the side of the petri dish and placed enzyme side down on the stain. Most enzymes required incubation at 37°C. Continuous monitoring was done and the bands marked as soon as they appeared. An ultra violet light was used for band monitoring whenever required (See Table 4).

To stop the reaction, membrane plates were placed in 5% glacial acetic acid for 10 seconds, rinsed with tap water, and allowed to air dry.

Selection of controls

The controls are just isolates of Leishmania that have been identified to the subspecific level, by CAE, and their enzyme "profile" known. Control isolates listed in Table 2 have been identified in a previous study by Kreutzer et al 1983. However, as new isolates from Table 1 were identified to the subspecific level, they were also used as control.

A control is always run adjacent to a new isolate to show similarity or differences to each other.

Calculations of similarity percentages

Once a new isolate was run for the 27 enzyme systems paired with a control or another isolate of the same group, the percent similarity between them was calculated using the following formula:

$$\text{Percent enzyme profile identical} = \frac{\text{(total isozyme systems in which identically migrating bands were produced)}}{\text{(total isozyme systems which produced a band for both isolates)}} \times 100$$

CHAPTER III

RESULTS

The twenty-seven enzyme systems used in electrophoresis are listed in Table 3. Most of them were previously used by Kreutzer and Christensen (1980) and Kreutzer et al (1983), and two new systems have been introduced, Alkaline phosphatase (ALF) and Glutathione reductase NAD (GSR_2). Some modifications have been performed on GPI, GSR_1, ICD, ME, MPI, and 6-PGDH so that they could be run under field conditions. Magnesium chloride ($MgCl_2$) was removed from the components of the cell buffer and stain, so that no "wet" element is present and packaging the dry chemicals in a mini-test kit could be done. Addition of only distilled water to the modified cell buffer, reaction buffer and stain will give the molarity and pH required. Despite the changes, bands obtained using these new systems were in agreement with the ones from the previously described systems.

The isolates listed in Table 1 were compared for similarities with a control from Table 2 (that have been identified previously by Kreutzer et al (1983) using the same technique) and in some cases with each other. Most of the isolates were run for twenty-six enzymes (GSR_2 was developed after most of the samples had already been identified), and since ACP, AK, FK, and FUM could be the result of two different loci, we have a total of thirty genetic loci for comparison purposes. Identity within an enzyme system was accepted when two dark bands from two different isolates migrated identically. Polymorphism which can account for 21% (Kreutzer et al 1983) was taken into consideration and fast, medium or slow was recorded (fast being the most anodal and slow the most

cathodal). The diagrammatic representations of the enzyme systems used, except for ALP, are shown in figure 1-26. Some enzymes are of greater identification value than others; however, the full complement of the enzyme systems is needed for a more accurate characterization.

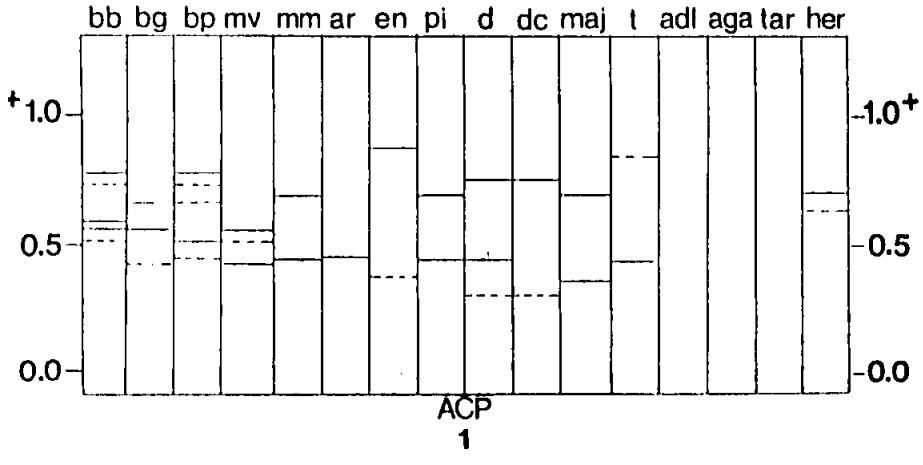
The lack of results for the twenty-seven enzymes with each isolate was due to both the weakness of some enzymic extract, low activities (ADH, ACP, ALP, HK) or "temperamental" development (ALP, GUK, HK) of some enzymes and the failure to have the volumes of isolates required for characterization with the wide range of enzymes that is now available for study (Miles et al 1979).

The percent similarity of the isolates from Table 1 to the control and in some instances between each other were calculated and recorded in Tables 5-12. Some isolates, such as L. aristedesi, L. m. enriettii, L. m. pifanoi, L. adleri, L. agamae and L. tarentolae, that had no previous controls, since they were run for the first time, have their diagrammatic representations drawn along with the other subspecies in fig. 1-26.

Most isolates produced bands of activity for all systems; however, some although active for most systems did not produce a band for one or more enzymes. L. m. enriettii, WR516 and WR529, did not show any band for 6-PGDH and the L. major isolates did not show bands for LDH: therefore, the allozymes for these specific enzymes might be null (Kreutzer et al 1983).

Many isolates produced more than one band of enzyme activity (fig 1,2,6,7,8,12,17,20). These could be isozymes but more data are needed. In certain of the Leishmania isolates which produced multiple bands, one of them was dark and the other light (fig. 1,2,7,12,17,20).

Fig. 1-26: Diagrammatic representations of the electrophoretic patterns of 26 enzyme/substrate systems from 16 species and subspecies of Leishmania. Numbers at the sides of each figure represent the mobilities of the enzymes. 0.0 is the origin on the cellulose acetate plate. Anode is at the top of each figure. Broken lines indicate lightly staining bands. See Table 3 for complete names of enzymes. bb, L. braziliensis braziliensis; bg, L. b. guyanensis; bp, L. b. panamensis; mv, L. mexicana venezuelensis; mm, L. m. mexicana; ar, L. m. aristedesi; en, L. m. enriettii; pi, L. m. pifanoi; d, L. donovani; dc, L. d. chagasi; maj, L. major; t, L. tropica; adl, L. adleri; aga, L. agamae; tar, L. tarentolae; her, L. hertigi. *No bands appeared.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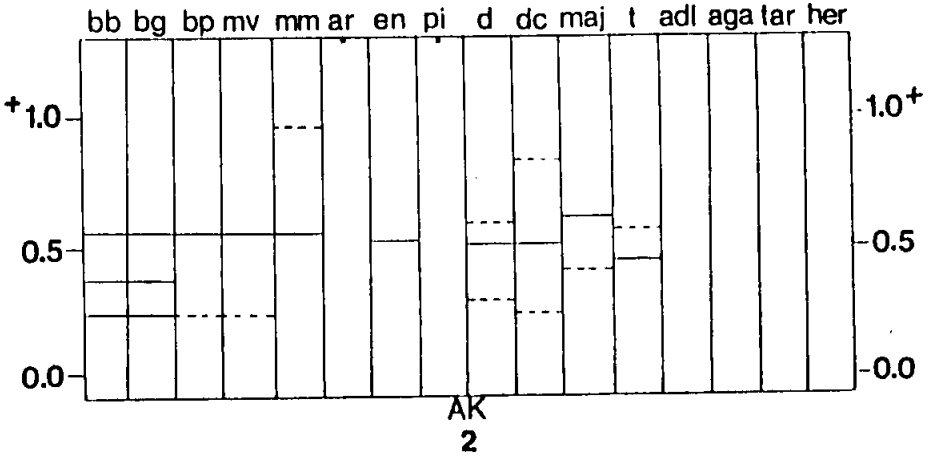
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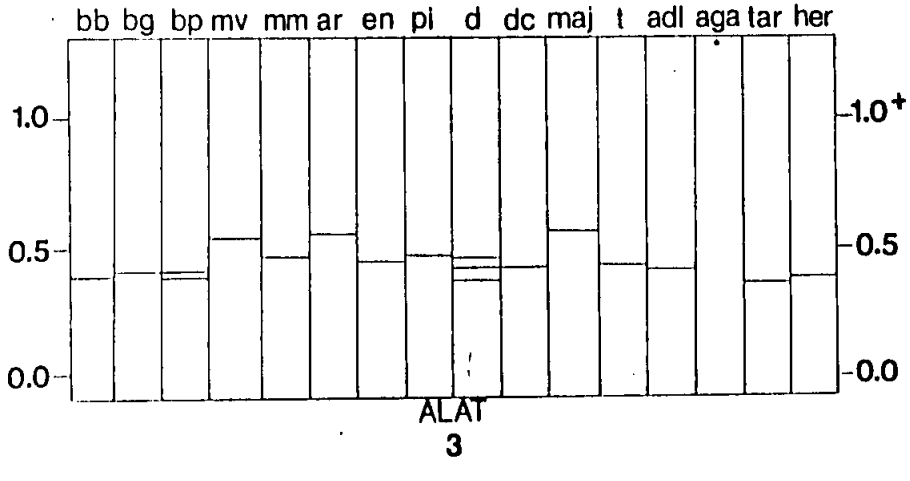
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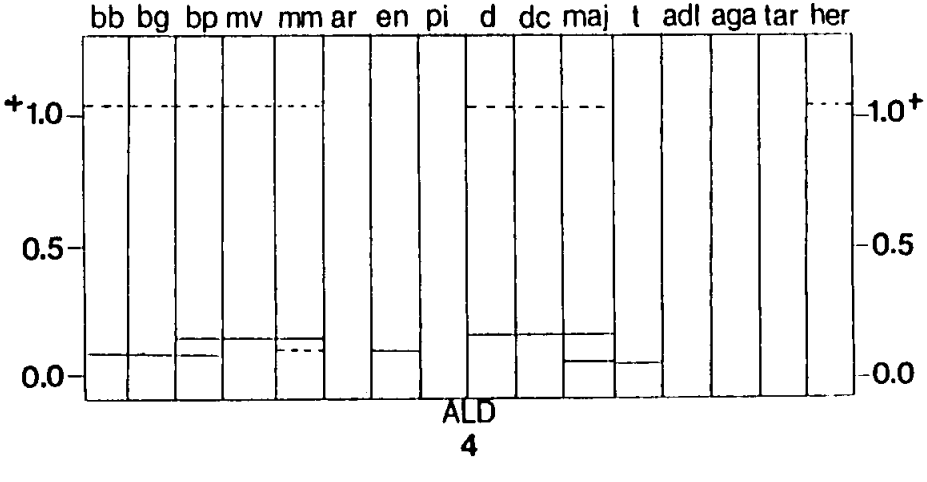
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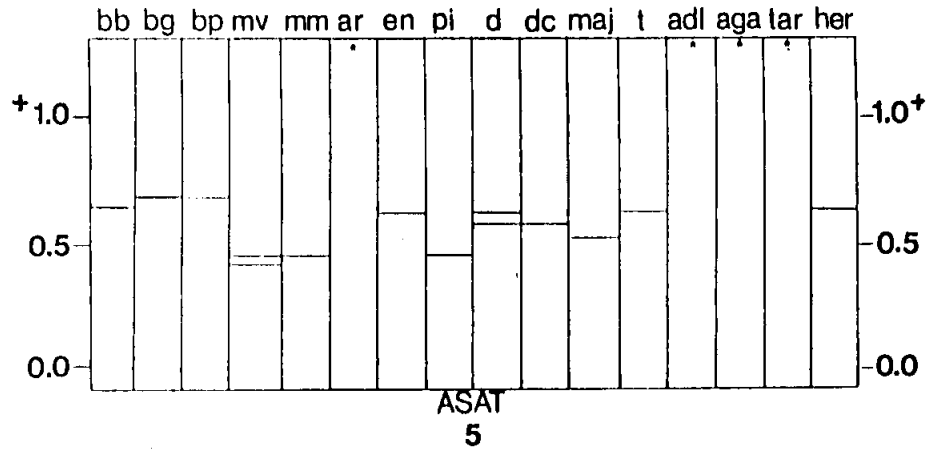
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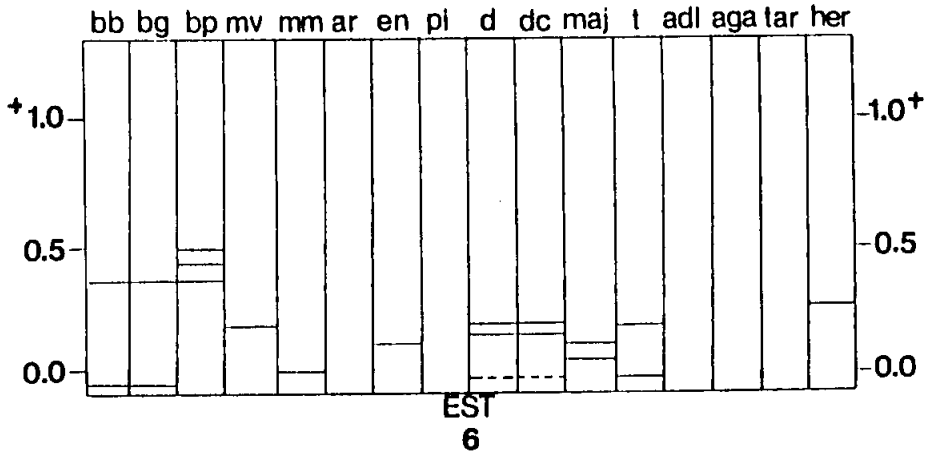
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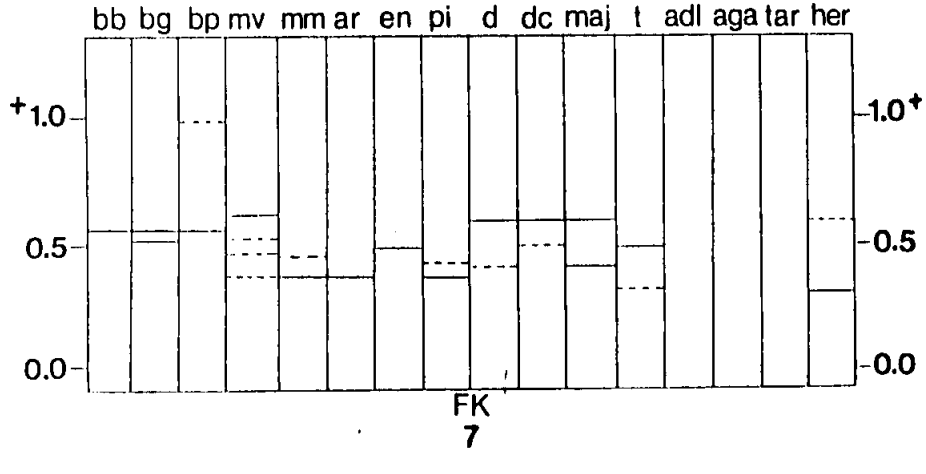
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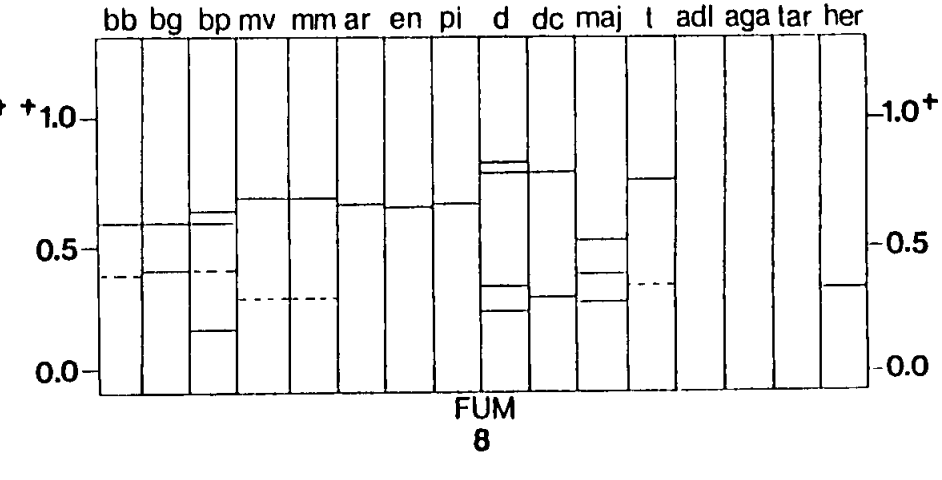
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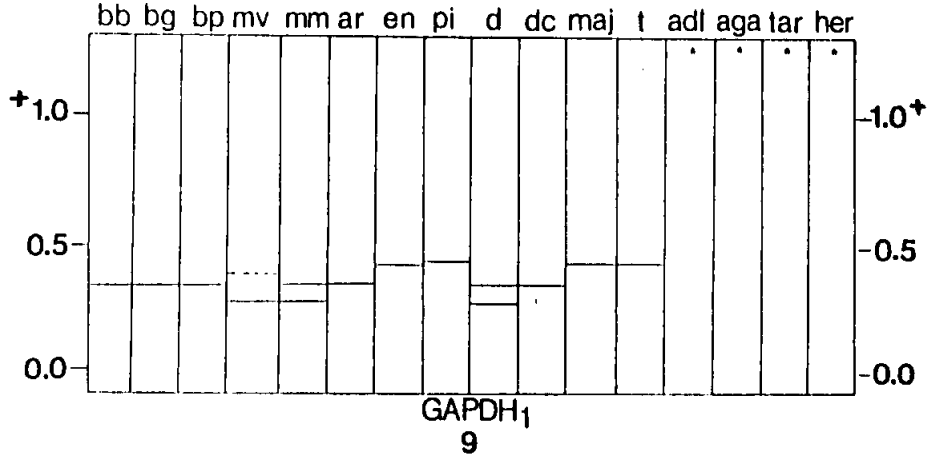
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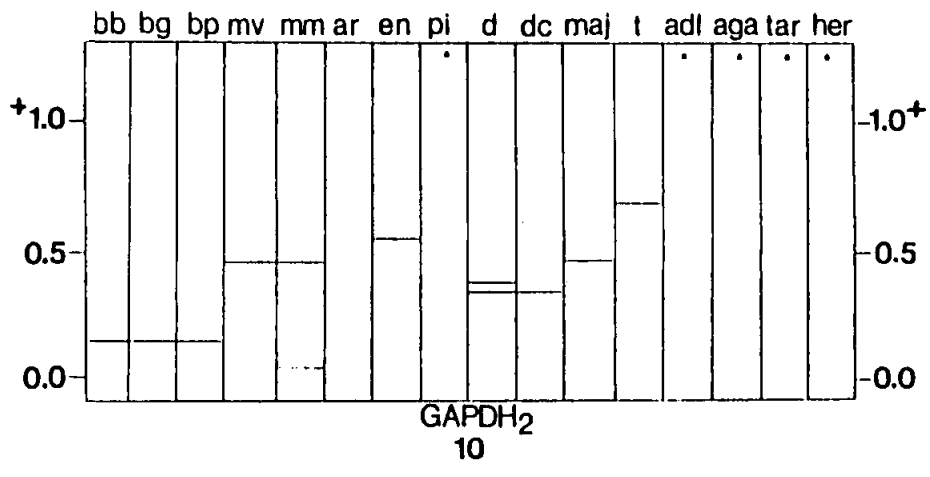
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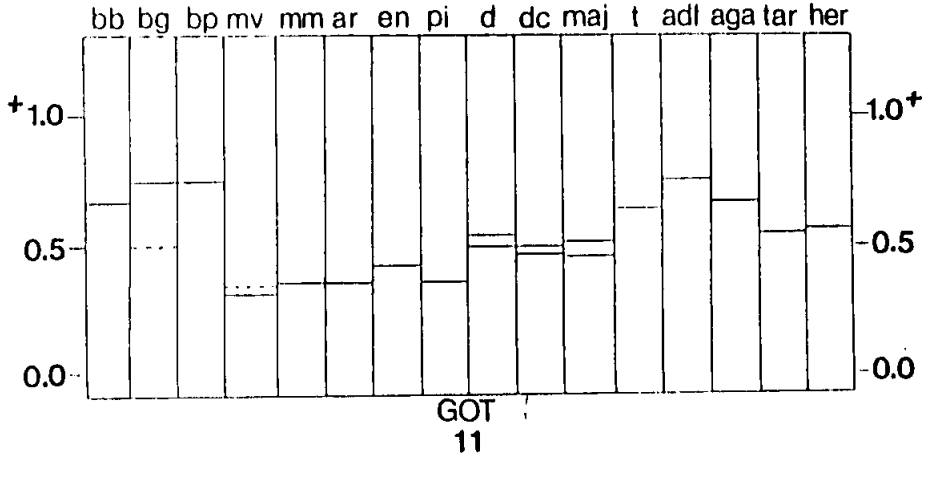
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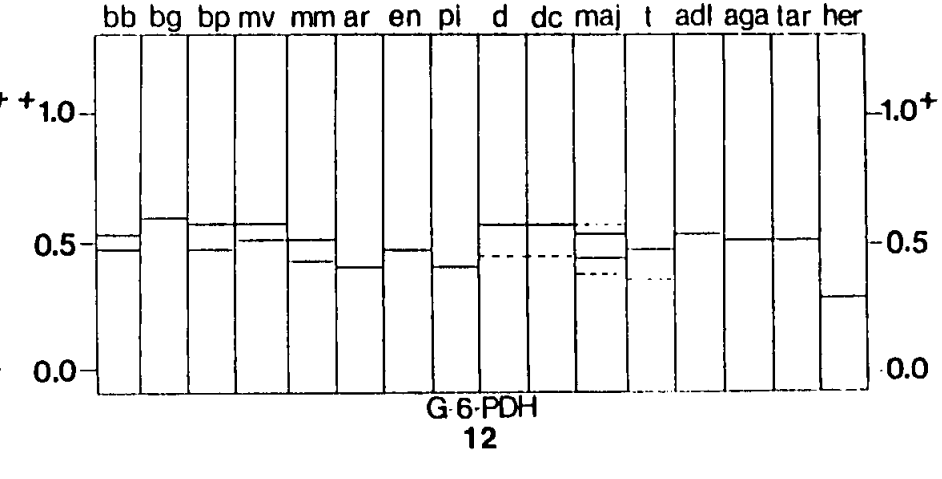
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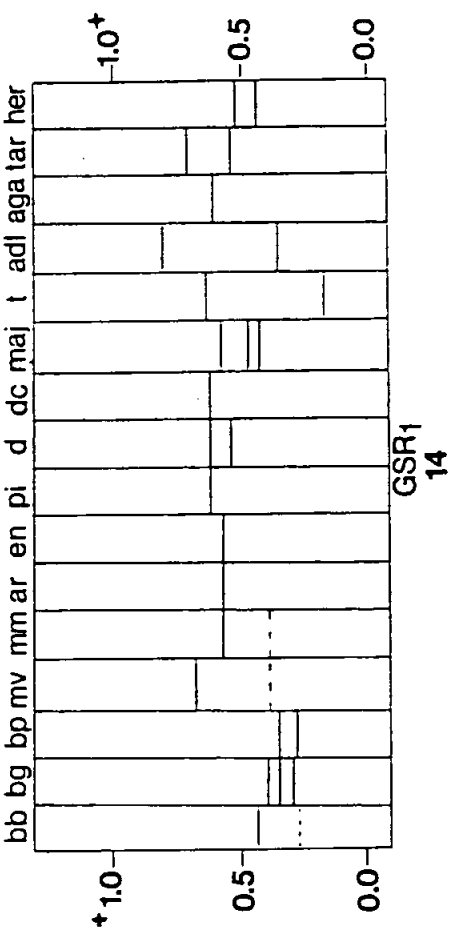
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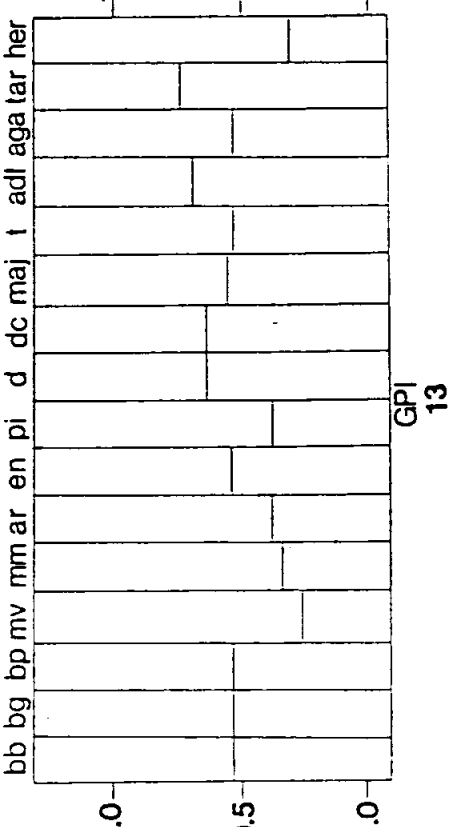
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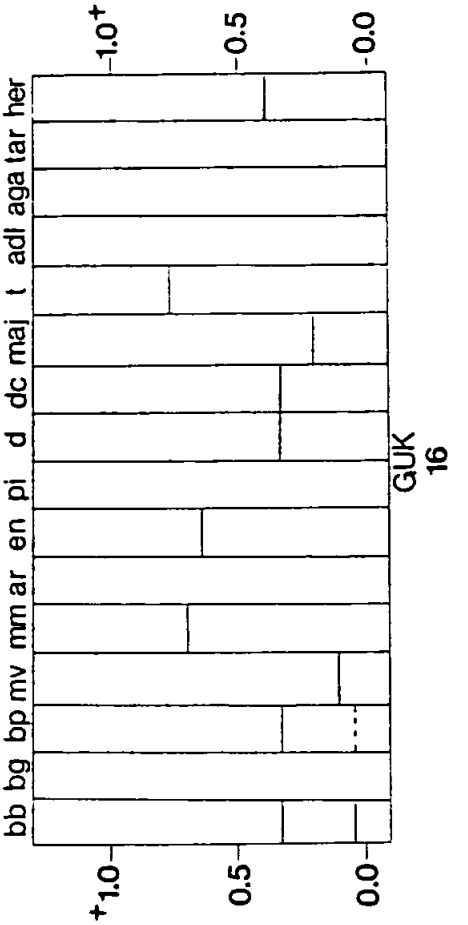
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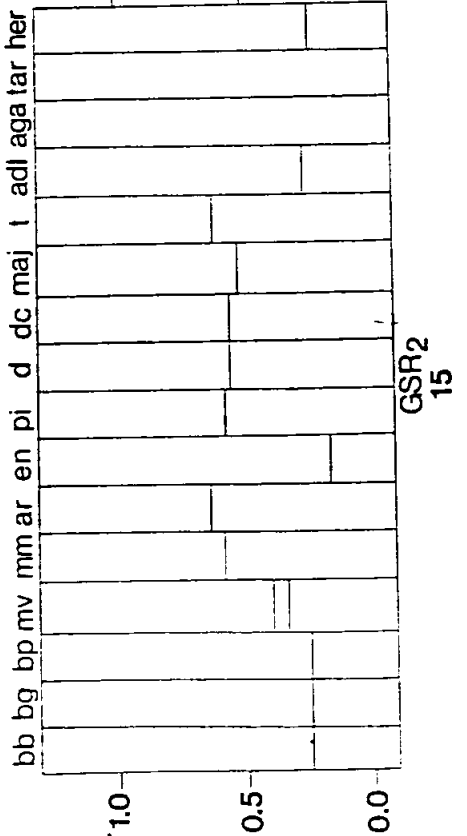
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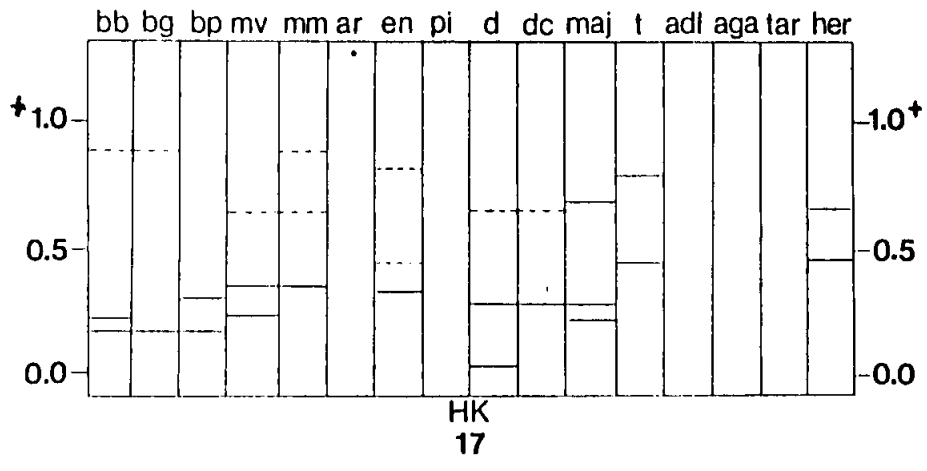
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HK
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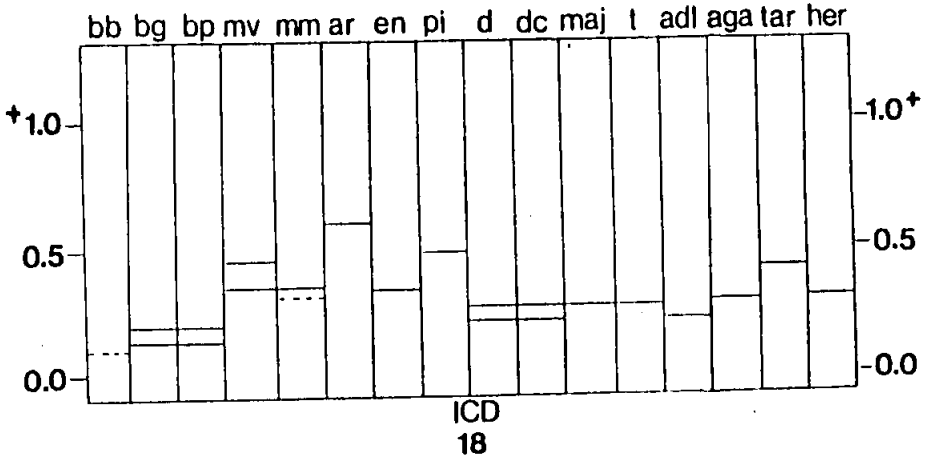
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ICD
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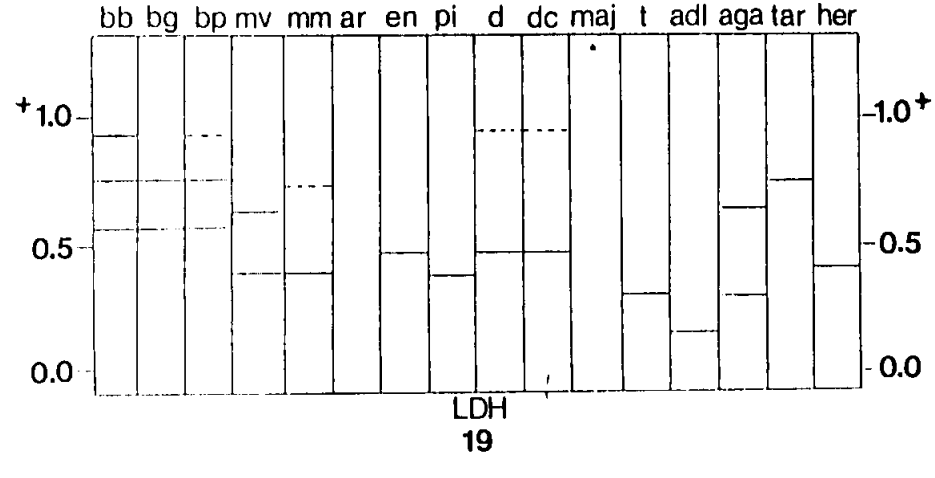
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LDH
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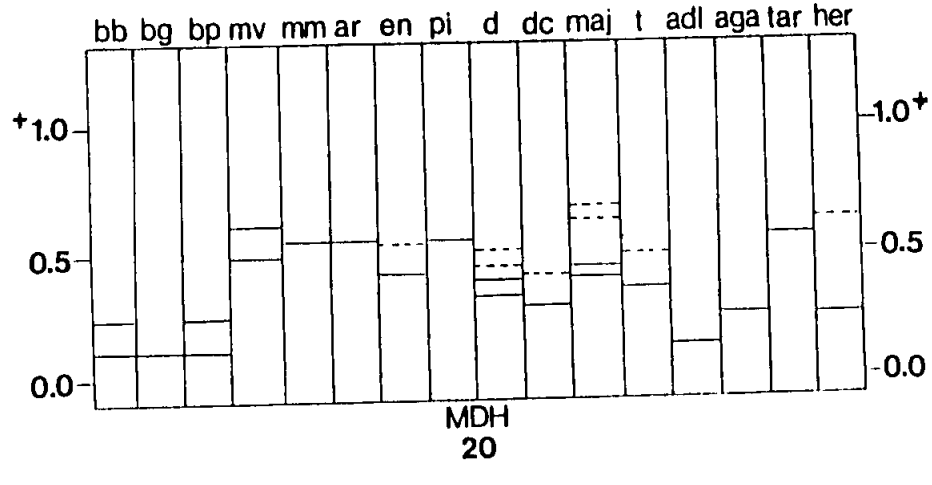
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0.0
MDH
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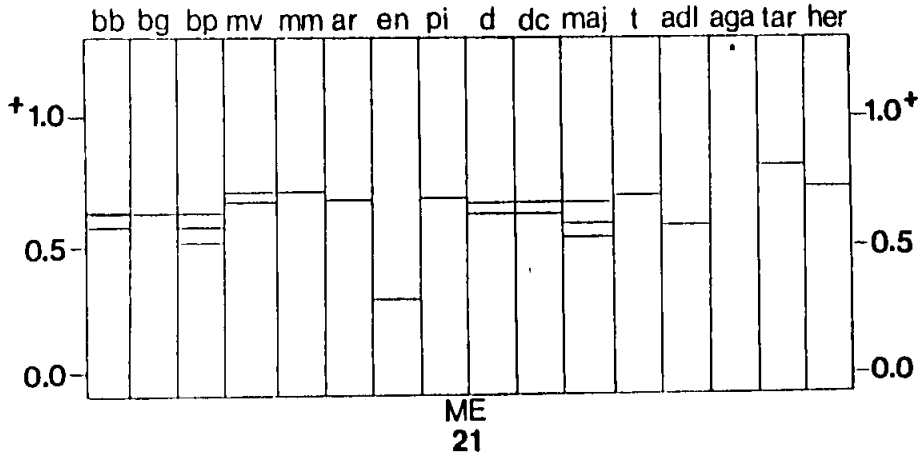
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0.0
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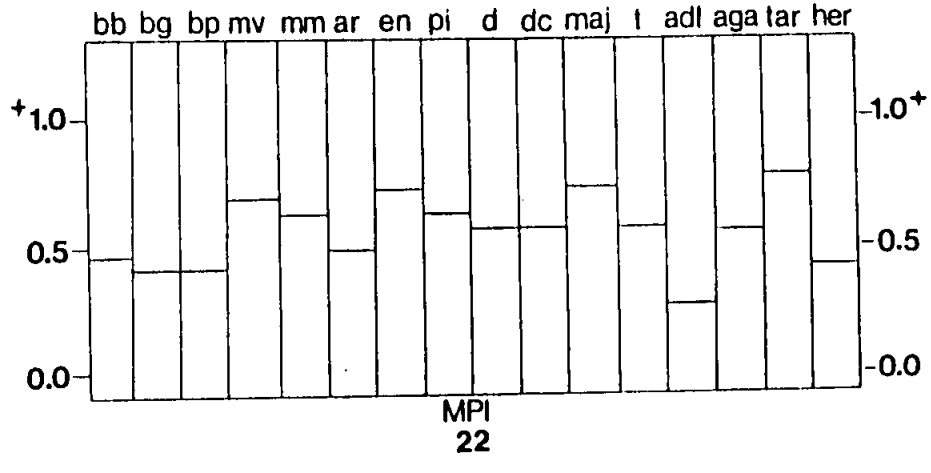
0.5
0.0
ME
21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0.0
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0.5
0.0
MPI
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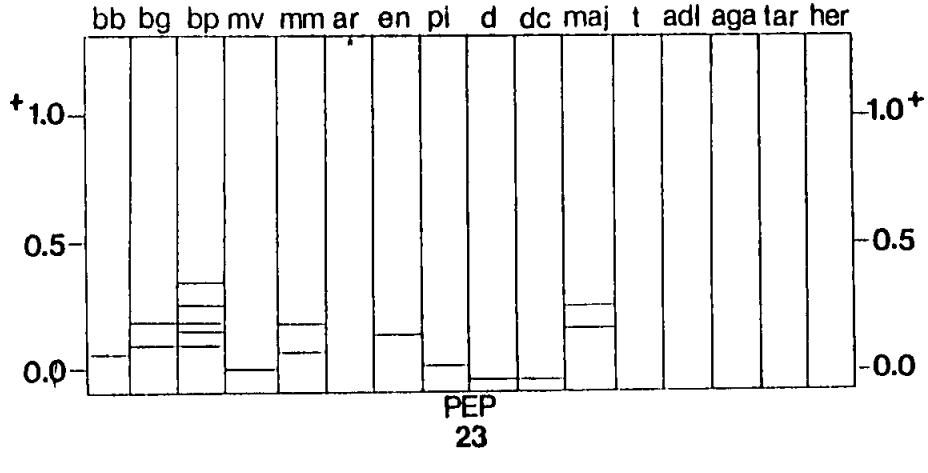
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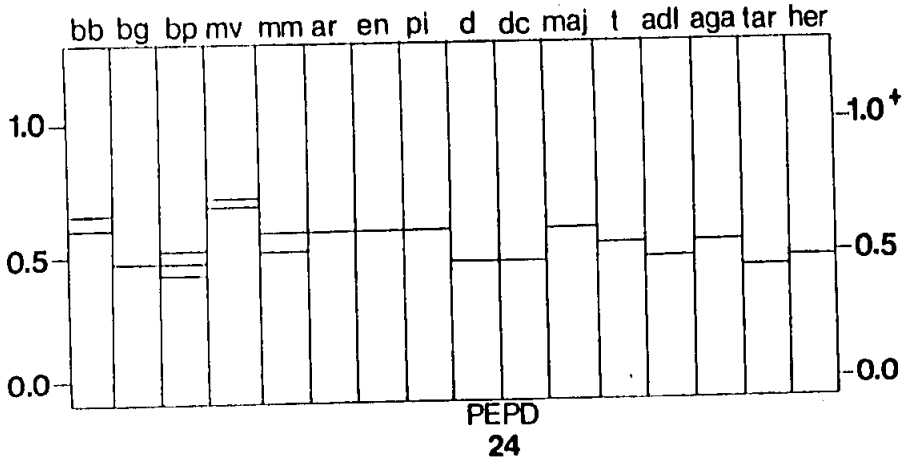
PEP
23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0.0
1.0+
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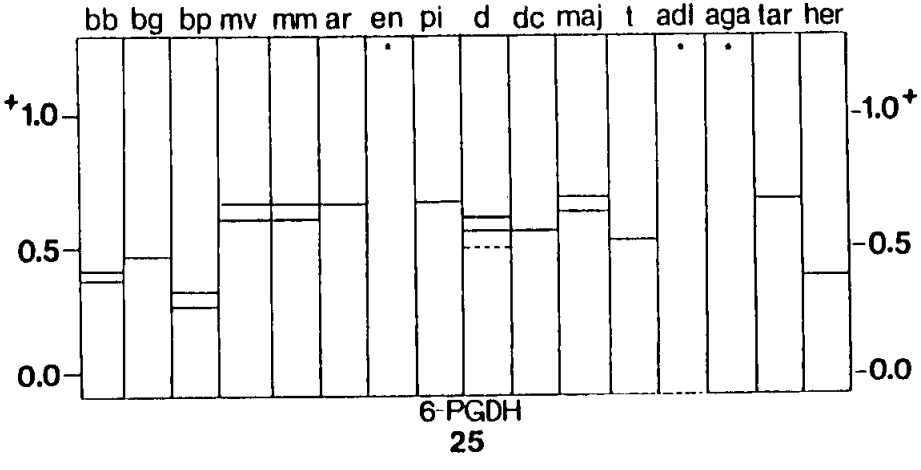
0.0
PEPD
24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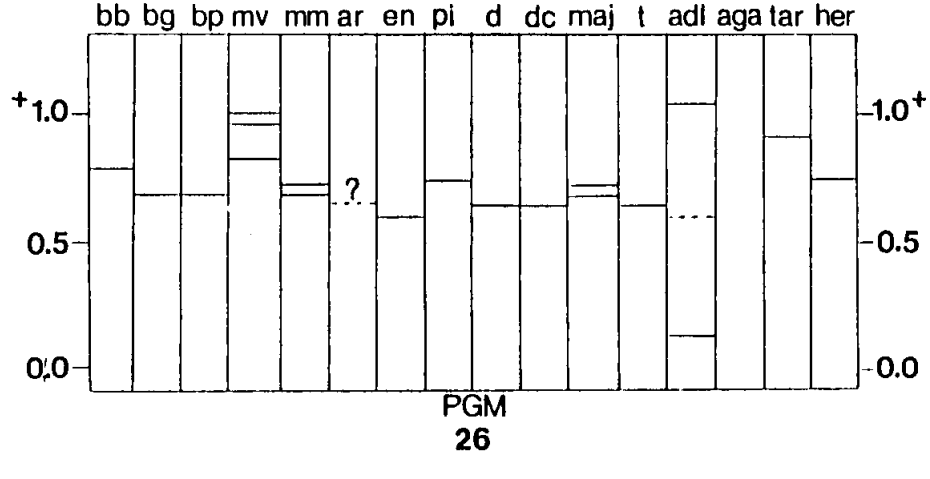
0.5
0.0
1.0+
0.5
0.0
6-PGDH
25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1.0
0.5
0.0
1.0+
0.5
0.0
?
PGM
26

Preliminary studies suggest that the isozyme which produces the dark band might be associated with the mitochondria and the light one is cytoplasmic (Kreutzer et al 1983). Four of the enzyme systems, ACP, AK, HK and FUM regularly produced at least two dark bands for each isolate.

The percent similarity within the L. braziliensis group, L. b. braziliensis and L. b. panamensis is seen in Tables 5, 6, and 12. These subspecies have a high similarity percentage, over 50%, between each other but less than 15% to any of the other groups. Since WR359, WR410, WR508, WR540, WR541, WR542, WR545 and WR557 were more closely related to WR294, the control for L. b. braziliensis, with over 90% similarity, they were identified as belonging to this subspecies (Table 5 and 6).

Within the L. b. panamensis type, the WRAIR isolates WR491, WR492, and WR493 were 100% identical to each other as well as were WR526 and WR539 (Table 12). The rest of the samples were over 80% similar to each other and to the control (Table 5).

For the L. mexicana profile, many subspecies are differentiated and identified by electrophoresis. The L. m. mexicana isolates WR347, WR524, WR531, WR585 and 019 from Columbia were over 75% identical to each other and to the control WR225 (Table 7 and 12). While the isolates WR348, WR381, WR411, WR447, WR453, WR457, WR468, WR548 and WR549, as well as all the controls listed as L. m. venezuelensis, have a specific enzyme "profile" with over 75% similarity to each other (Tables 7 and 8), but only 40% similarity to WR225 (L. m. mexicana). The L. m. venezuelensis enzyme "profile" was also 42% similar to L. m. aristedesi and 48% identical to L. m. pifanoi while only 20% similar to L. tropica and less than 15% with the other groups. Because of its similarity to L. mexicana it

is considered part of the mexicana complex but of a specific subspecies. The enzymes GPI and MPI could be sufficient to identify this subspecies.

The other L. mexicana subspecies did not have a large number of isolates for an accurate characterization and more information is still needed. L. m. aristedesi, WR481, is 52% similar to WR225, whereas L. m. pifanoi is 58% similar to WR225, while L. m. enriettii, WR516 and and WR529, are only 35% similar to WR225 (Table 7).

Within the L. donovani complex in table 9, the old world (L. donovani) and the new world (L. d. chagasi) visceral types have been found to be over 75% similar. The enzyme MDH could be the enzyme for separating the old and new world subspecies, since the L. d. chagasi have a more cathodal migration than the L. donovani. Also, GOT and ICD could be probable enzyme systems for separating these two subspecies, but polymorphism occurring within their enzyme "profile" (fig. 21) complicates the identification. More isolates of the two types are required for a more accurate identification of the polymorphism.

The WRAIR isolates WR308 and WR309 previously identified as L. tropica (Kreutzer et al 1983), are now identified as L. major. A different "profile" for L. tropica has been described with the following isolates: WR581, WR582, WR583 and WR584. The enzyme "profile" for L. major (Table 10) includes WR547, WR551, WR552, WR558, WR559, WR586 and WR587 with WR308 and WR309 as controls. The L. major and L. tropica have been found to have less than 30% similarity but are still part of the same complex.

The isolates listed as Leishmania sp., WR316, WR553, WR554, and WR544, or as unknowns WR177, WR440, and WR495, have low similarities with any of the above "profiles." WR177 has 30% similarity with WR005, an

L. b. panamensis control, 25% with WR294 and 33% with WR359, two L. b. braziliensis isolates, and lower similarities with the other groups (Tables 5 and 11). WR316 has 50% similarity with L. mexicana subspecies (Table 11). WR553 and WR554 while being 100% identical to each other (Table 12) are less than 15% similar to any of the other groupings. A similar situation is present for WR544 from Belize, it is less than 15% similar to each type. Finally, WR440 and WR495 are 100% identical to each other (Table 12), but less than 15% similar to any of the groups (Table 9). With the previous unknowns more information is needed to be able to identify them as Leishmania subspecies.

Furthermore, L. adleri, L. agamae, L. tarentolae and Herpetomonas (WR523), have an enzyme "profile" of their own (fig. 1-26). L. adleri is 45% similar to the L. braziliensis profile, 21% to L. mexicana and 36% to L. donovani. L. agamae is 40% similar to L. braziliensis, 30% to L. mexicana and 20% to L. donovani. L. tarentolae is 23% similar to both L. braziliensis and L. mexicana "profiles" and only 15% to L. donovani. However, L. adleri and L. agamae are 60% similar, while L. tarentolae is less than 15% to either one. Herpetomonas is 27% similar to WR225 but less than 15% similar to any of the other complex, which is understandable since it is not a Leishmania subspecies.

In fig. 27, a dendogram of the grouping of Leishmania subspecies according to their similarity percentages. The five different Leishmania complex are seen with the WRAIR isolates belonging to each subspecies.

PERCENT SIMILARITY BETWEEN PAIRS OF ISOLATES AND CONTROL ISOLATES IN THE L. RRAZILIENSIS* PROFILE. THESE ISOLATES HAVE LESS THAN 15% IDENTITY WITH ANY OF THE OTHERS.

Lbp 111[a]	Lbp 176	Unkn+ 177	Lbp 241	Lbb 294	Lsp 316	Lbh 359	Lbb 410	Lbp 470	Lbp 487	Lbp 491	Lbb 508	Lbp 526	Lbp 535	Lbp 578	
96[b]	-	30	85	-	-	47	43	96	-	-	-	-	-	-	008
	80	-	-	50	28	50	52	-	80	-	50	-	-	-	111
		25	-	-	-	82	92	48	-	40	80	40	40	50	294
										86	-	86	89	-	128
											25	-	-	-	177
												96	98	89	491
													98	-	526
														87	470

*Lbp, Leishmania braziliensis panamensis; Lbb, L. b. braziliensis; Lsp., Leishmania sp.

+ Unkn, WR177 listed as unknown.

a WRAIR isolates.

b Percent enzyme profile identical-(total isozyme systems in which identically migrating bands were produced) /(total isozyme systems which produced a band for both isolates) X 100.

PERCENT SIMILARITY BETWEEN PAIRS OF ISOLATES AND CONTROL ISOLATES WITH THE
L. BRAZILIENSIS BRAZILIENSIS ISOLATES FROM BELIZE

Lbb 359[a]	Lbb 410	Lbb 540	Lbb 541	Lbb 542	Lbb 557	Lbb 543	Lbb 545	
82[b]	92	96	91	92	95	94	94	294
			93	93	94	93	93	540
				94	90	98	94	541
					95	96	92	542
						98	100	557
							96	543
								545

a WRAIR isolates.
b Percent (enzymeprofile identical-(total 'isozymesystems in which identically migrating bands were produced)/ (total isozyme systems which produced a band for both isolates) X 100.

TABLE 7

PERCENT SIMILARITY BETWEEN PAIRS OF ISOLATES AND CONTROL ISOLATES IN THE L. MEXICANA* PROFILE. THESE ISOLATES HAVE LESS THAN 15% IDENTITY WITH ANY OF THE OTHERS.

Lme 516[a]	Lmm 225	Lmm 019[b]	Lmv 183	Lmv 338	Lmv 381	Lmv 447	Lmv 453	Lma 481	Lmp 528	Lmm 585	
	35[c]	-	-	-	-	-	-	-	-	-	516
		78	-	57	50	-	-	52	58	88	225
						-	-	-	-	90	019
					80	-	-	57	-	-	183
					69	-	-	-		-	338
						64	67	48		-	381
								55		-	447
								52		-	453

* Lme, L. mexicana enriettii; Lmm, L. m. mexicana; Lmv, L. m. venezuelensis; Lma, L. m. aristedesi; Lmp, L. m. pifanoi.

a WRAIR isolates.

b 019 is an isolate from the NIH in Columbia.

c Percent enzyme profile identical-(total isozyme systems in which identically migrating bands were produced)/(total isozyme systems which produced a band for both isolates) X 100.

PERCENT SIMILARITY BETWEEN PAIRS OF ISOLATES IN THE
L. MEXICANA VENEZUELENSIS PROFILE.

Lmv 140[a]	Lmv 183	Lmv 411	Lmv 447	Lmv 453	Lmv 468	Lmv [illegible]	Lmv 338	Lmv 548	Lmv 549	
	85[b]	-	67	[illegible]0	63	[illegible]2	72	-	-	140
		-	77	[illegible]	75	-	83	-	-	183
		8[illegible]	-	-	-	88	-	95	94	348
			-	-	-	93	-	88	88	411
				81	76	-	83	-	-	447
				76	-	100	-	-	-	457
					83	7[illegible]	70	-	-	453
						3[illegible]	-	-	-	225
							80	-	-	468
								92	94	527
									95	548
										549

WRAIR isolates.
Percent enzyme profile identical-(total isozyme systems in which identically migrating bands were produced)/ (total isozyme systems which produced a band for both isolates) X 100.

TABLE 9

PERCENT SIMILARITY BETWEEN PAIRS OF ISOLATES AND CONTROL ISOLATES IN THE L. DONOVANI PROFILE. THESE ISOLATES HAVE LESS THAN 15% IDENTITY WITH ANY OF THE OTHERS. ALSO INCLUDED ARE AN UNKNOWN ISOLATE AND AN L. TROPICA ONE.

Ld 130[a]	Ldc 285	Ldc 317	Ldc 341	Ld 425	Unkn 440	Ldc 520	Ldc 555	Lt 581	
81[b]	89	98	94	81	-	-	-	-	116
	94	92	94	79	-	-	-	-	130
		94	92	90	-	-	-	-	285
			94	94	-	-	-	-	317
				94	-	-	-	-	341
					4	84	94	25	425

*Ld, L. donovani; Ldc, L. d. Chagasi; Unkn, unknown; Lt, L. tropica.

a WRAIR isolates.

b Percent enzyme profile identical-(total isozyme systems in which identically migrating bands were produced) /(total isozyme systems which produced a band for both isolates) X 100.

TABLE 10

PERCENT SIMILARITY BETWEEN PAIRS OF ISOLATES AND CONTROL ISOLATES IN THE L. MAJOR PROFILE. THE ISOLATES HAVE LESS THAN 15% IDENTITY WITH ANY OF THE OTHERS

Lmaj 308[a]	Lmaj 547	Lmaj 551	Lmaj 552	*Lmaj* 558	Lmaj 559	Lmaj 586	Lmaj 587	
	90[b]	90	90	86	87	-	-	308
		100	100	93	95	-	-	547
			100	92	95	-	-	551
				92	95	-	-	552
					92	-	-	558
						98	98	559
							100	586
								587

a **WRAIR** isolates.

b Percent enzyme profile identical = (total isozyme systems in which identically migrating bands were produced)/ (total isozyme systems which produced a band for both isolates) x 100.

TABLE 11

PERCENT SIMILARITY BETWEEN UNKNOWNS ISOLATES, *LEISHMANIA SP.*, *HERPETOMONAS* AND CONTROL ISOLATES*

Unkn 440[a]	Lmv 527	Unkn 177	Herp 523	Lsp 316	Lbb 359	Lmm 225	Lbp 241	Lt 581[b]	Lmaj 308	Lmv 338	Ld 425	
-	-	-	-	-	-	-	-	-	-	-	4	440
	20[c]	-	25	15	33	9	14	19	13	10	-	177
					18	50	28	38	31	19	-	316
						15	35	27	32	24	-	359

*Unkn, unknowns; Lmv, *L. mexicana venezuelensis*; Herp, *Herpetomonas*; Lsp. *Leishmania sp.*, Lbb, *L. braziliensis braziliensis*; Lmm, *L. m. mexicana*; Lbp, *L. b. panamensis*; Lt, *L. tropica*; Lmaj, *L. major*; Ld, *L. donovani*.

a WRAIR isolates.

b *L. tropica* isolates, WR581, WR582, WR583, WR584.

c Percent enzyme profile identical-(total isozyme systems in which identically migrating bands were produced)/ (total isozyme systems which produced a band for both isolates) X 100.

TABLE 12

ISOLATES OF *LEISHMANIA SP.*[*] (440-495 ARE UNKNOWNS) WITH HIGH PERCENTAGE OF SIMILARITY BETWEEN PAIRS OR GROUPS OF THEM AND ARE NOT LISTED IN THE PREVIOUS TABLES.

ISOLATES NUMBER[a]	% SIMILARITY
L.b.p.: 491-492-493	100
L.b.p.: 492-556	100
L.b.p.: 526-539	100
L.b.p.: 008-246-282-360-380-442-446-470 475-505	100
L.b.p.: 008-111-003-004-111C-111LN-132 176-154-179-211-232-322-315-390 470	>90
L.b.b.: 545-557	100
L.m.m.: 225-127-347-524-531	>90
L.d.: 425-311	>90
L.maj.: 547-551-552	100
L.maj.: 586-587	100
L.t.: 218-581-582-583-584	100
L.t.: 218-031[b]	>90
L.m.e.: 516-529	100
L.sp.: 553-554	100
Unk.: 440-495	100

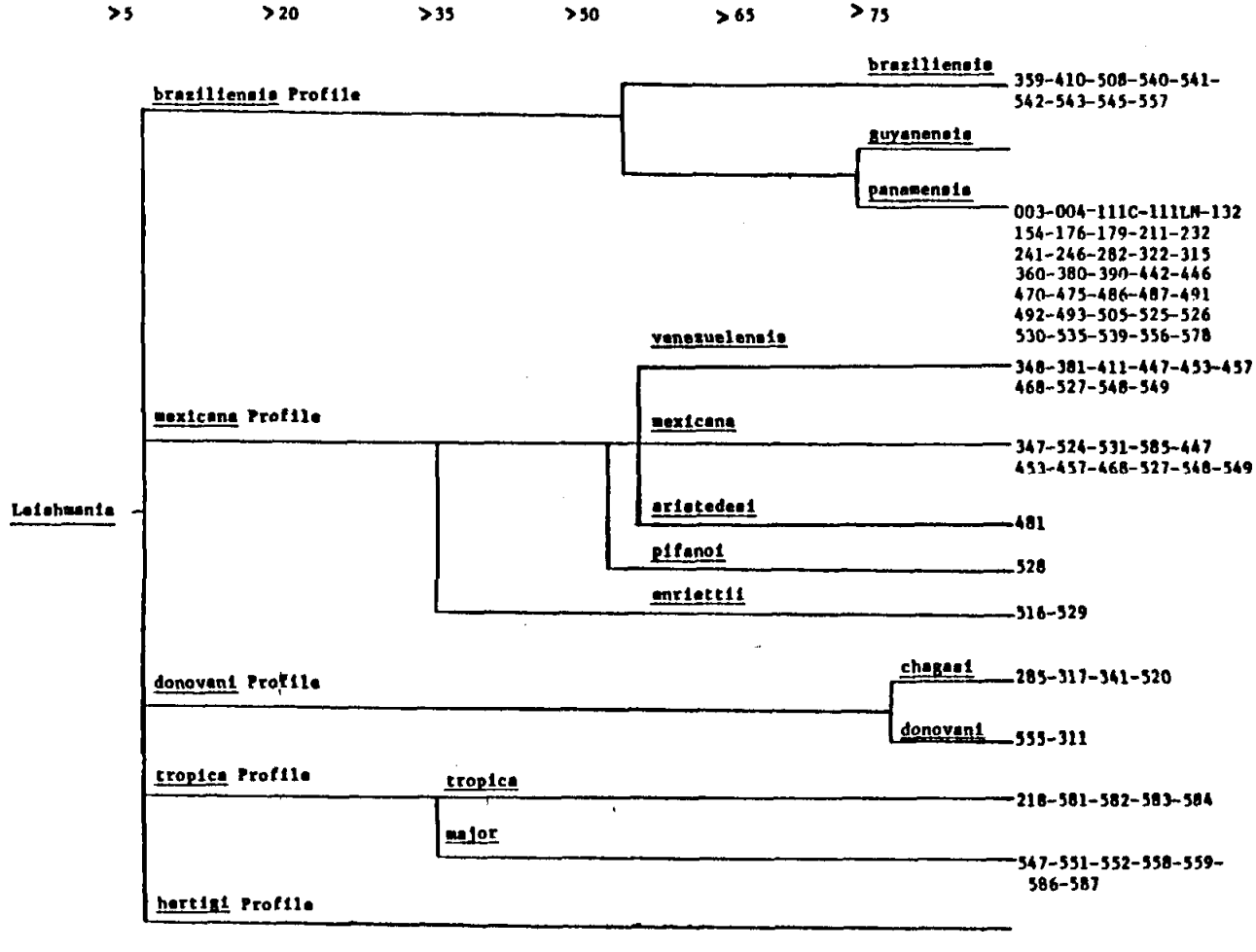
[A] Lbp, *L. braziliensis panamensis*; Lbb, *L. b. braziliensis*: Lmm, *L. mexicana mexicana*; Ld, *L. donovani*; Lmaj, *L. major*; Lt, *L. tropica*; Lme, *L. m. enriettii*; Lsp, *Leishmania sp.*: Unkn, Unknowns.

a WRAIR isolates.

b 031 is an isolate from the NIH in Columbia

FIGURE 27

GROUPING OF ISOLATES ACCORDING TO THEIR LEVELS OF ENZYME PROFILE SIMILARITIES. PROFILES OF ISOLATES GROUPED AT THE RIGHT ON THE SAME LINE ARE ABOUT 75% OR MORE IDENTICAL. SEE TABLE 1 FOR ISOLATE INFORMATION.



CHAPTER IV

DISCUSSION

Populations belonging to different species almost invariably show considerably more genic differences than do conspecific populations. Electrophoretic techniques yield very precise data on genetic contents of organisms. The large biochemical differences between species makes electrophoretic techniques of great value in describing and identifying members of different subspecies that are morphologically indistinguishable (Avise 1975).

Variations in the electrophoretic mobility of enzymes have been used increasingly in the differentiation of leishmania1 strain (Brazil 1977), and up to twenty-five enzymes have been used before (Kreutzer and Christensen 1978, and Kreutzer *et al* 1983). Kreutzer *et al* used only 44 isolates of various subspecies of Leishmania: however, with the increasing number of isolates run and identified, a more accurate "profile" of the different groupings as well as polymorphism present within each subspeciesis obtained.

Two new enzymes have been added to the already present twenty-five. Alkaline phosphatase (ALP), which is very weak and of little value in characterization, and Glutathione reductase NAD (GSR_2) (fig. 15), which produces good bands as well as good separation between the L. braziliensis and the L. mexicana subspecies, and also within the L. mexicana group, gives different migrations for L. m. mexicana, L. m. venezuelensis and L. m. enriettii. GSR_2 does not, however, differentiate between the L. m. mexicana, the L. donovani and the L. tropica, but

shows different levels between the three previous subspecies and L. major type which has a more cathodal band and could be identified using this enzyme (fig. 15).

Furthermore, modifications in the buffer/substrate components of GPI, GSR_1, ICD, MDH, ME, MPI and 6-PGDH systems, were introduced so as not to require a pH meter (Table 4). The addition of only distilled water to preweighed chemical mixtures will give the exact pH and molarity needed. These enzyme systems could be packaged ahead of time and used for mini-tests in field conditions so that no sophisticated equipment other than a power supply, a cell, a refrigerator, and in the case of MDH an ultra-violet light, would be necessary.

The seven previous enzymes are used in preliminary studies. However, an unknown isolate could be identified to the subspecific level with only three of them, GPI, MPI and 6-PGDH. Three subspecies of L. braziliensis can be separated by 6-PGDH and then separated from visceral and L. mexicana profile (fig. 25). MPI is able to differentiate two subspecies of L. braziliensis, four subspecies of L. mexicana from other visceral isolates, L. tropica and L. tarentolae (fig. 22), while GPI separates four subspecies of L. mexicana, L. tropica, visceral isolates, L. adleri and L. tarentolae (fig. 13). The other four enzyme systems ICD, GSR_1, MDH and ME are also very valuable in identification and should be run for confirmation in preliminary studies. GSR_2 could be added to the seven systems in the mini-test kit since its buffers do not need a pH meter. Despite the good identification obtained with only these eight enzymes, all the enzyme systems should be run for more accurate percent similarities, especially in the occurrence of polymorphism.

In the L. braziliensis group, 6-PGDH will differentiate between

the three subspecies L. b. braziliensis, L. b. guyanensis and L. b. panamensis, even though polymorphism is present but does not overlap (fig. 25). L. b. braziliensis is also separated from the other two subspecies by the following enzymes: ASAT, GOT, GSR_1, ICD, MPI, PEP, PEPD, 6-PGDH and PGM (fig. 5,11,14,18,20,23,24,55 and 26). The isolates WR540, WR541, WR542, WR543, WR545 and WR554 from Belize and WR359 and WR410 from Panama were identified as belonging to this subspecie. The Belize isolates had a high level of identity, over 90% between each other and the control WR294 (Table 6), and less than 70% with the other braziliensis subspecies (Table 5). The L. b. panamensis profile is very similar to L. b. guyanensis except for G6-PDH and 6-PGDH which differentiates them from each other (fig. 12,25). The L. b. panamensis isolates were from Panama except WR111C and WR111LN from Belize and WR390 from Brazil. The WR491, WR492, and WR493 isolates are from the same host and their similarity of 100% is expected.

For the L. mexicana group, the enzymes GPI, MPI, and 6-PGDH are sufficient to separate L. m. mexicana from L. m. venezuelensis (fig. 1-26). The L. m. mexicana isolates are from different areas, WR347 from Belize, WR524 and WR531 from Panama, 019 from Columbia and WR585 of unknown origin.

The L. m. venezuelensis group is very widespread and found in many countries including Costa-Rica, Dominican Republic, Belize, Panama, Peru, USA(Texas) & Venezuela(Table 1 & 2). To the thirteen L. m. venezuelensis isolates (including the controls), five different isolates, NIH/IS, NIH/EM, VA, Ba and OC previously identified by CAE as L. m. venezuelensis, were compared to show similarities within the group. Of the five isolates, three were from Venezuela and two from the Dominican

Republic and all five were diagnosed as DCL. So of the eighteen isolates now available, eight were diagnosed as DCL and ten as simple cutaneous. The Venezuela and Dominican Republic isolates had 90% to 100% subspecies identity, the Panama isolate was distinct from all others for two enzymes, the Peru isolate was distinct for one enzyme and the Belize and Peru isolates were more similar with each other than they were with the Venezuela, Dominican Republic or Panama isolates (Kreutzer et al, paper presented at meeting 1983). Despite few differences within certain enzymes, the causative agent of DCL has a distinct enzyme "profile" that is 40% similar to L. m. mexicana, including it as a subspeciesof the mexicana complex that could be L. m. venezuelensis and is referred to by this name throughout this paper. Among isolates of this subspecies polymorphism was noted for ALAT, ALP, ASAT, GOT, GUK, MDH and ME (fig. 3,5,11, 16,20 and 21). Also of importance in polymorphism is GSR_2 since four isolates from the Dominican Republic, WR336, WR453, WR468 and WR338 (no data were obtained from the other Dominican isolates) had a more cathodal band than all the other from different areas. This difference might be of value in showing polymorphism related to geographic areas.

The visceral Leishmania groups from the old world (L. donovani) and the new world (L. d. chagasi) are very similar in their enzyme "profiles" (fig. 1-26) and identification of these two groups is complicated by polymorphism. Further information is needed. The enzymes that might differentiate them are ICD, GOT and MDH (fig. 18,11,20), but until now they are mainly differentiated by their country of origin; In this study the L. donovani from the old world were isolated from Khartoum and Kenya while the L. d. chagasi from the new world, from Brazil and Panama.

The L. major and L. tropica groups have been restricted only to the old world. However from the results obtained, most of the L. major isolates WR547, WR551, WR552, WR558 and WR559 were from Kenya while WR586 and WR587, of unknown origin from South America, were previously identified as L. braziliensis type from the new world, but have been currently identified by CAE as L. major. It is possible that either L. major is present in the new world or that contamination in the laboratory occurred. In the case of L. tropica, WR581, WR582, WR583 and WR584 are from the old world, but WR218 is from the Canal Zone and 013 from Columbia. Again, this situation shows either the presence of L. tropica in the new world or that contamination occurred.

The remaining of the L. mexicana group, L. m. aristedesi WR481, L. m. pifanoi WR528, and L. m. enriettii WR516 and WR529 had only one or two isolates run and their profiles are drawn with the others (fig. 1-25). They were related to L. m. mexicana (Tables 7 & 11) but not of the same subspecies. However, more information is needed on their subgroups.

The three lizard Leishmania subspecies, L. adleri, L. agamae and L. tarentolae, are less than 40% similar to any of the other groups. Their enzyme "profiles" are drawn for some of the systems (fig. 1-25). The entire profile is not present because of the small volume of the isolate which was not sufficient for the enzyme range available for study.

Isolates listed as Leishmania sp. or unknown could not be classified in any of the above subspecies, because of their low similarity to any of the identified types. From the ones classified as Leishmania sp., WR316 is 50% similar to the L. m. mexicana, so could be a subspeciesof the mexicana complex but do not belong to any of the types

for which we have an enzyme "profile." WR544, also known as Bel21C from Belize did not show similarity to any of the known subspecies and is still listed as Leishmania sp. WR553 and WR554 from Ecuador, were identical to each other and probably from the same source, but they were less than 15% similar with any of the other groups. The unknown, WR177 from Brazil has 33% similarity with WR355, an L. b. braziliensis isolate, 30% similarity to WR008 and less with the other groups, so is still unidentified. WR440 and WR495 were 100% identical to each other. They are from Kenya and diagnosed as visceral, but they had only 4% identity with the old world visceral control WR425. Therefore, it is still an unknown. The three unknowns could either be a Leishmania subspecies not yet identified by CAE or could also be another genus than Leishmania, since CAE is of little value at the generic level. Herpetomonas isolate from Brazil is 27% similar to WR225 (Table 11), but is not a Leishmania subspecies.

Therefore, with the increasing number of isolates run, a better and more precise identification process is evolving. As the number of isolates identified increases, the number of subspecies within the same area is increasing, as well as the number of different countries where a specific subspecie is found. L. b. braziliensis, L. b. panamensis and L. d. chagasi have been identified from Brazil. Whereas, in Panama L. b. braziliensis, L. b. panamensis, L. m. mexicana, L. m. venezuelensis, L. m. aristedesi and L. d. chagasi have been isolated and in Belize, L. b. braziliensis, L. b. panamensis, L. m. mexicana and L. m. venezuelensis have been identified. So it is clear that the country of origin as a diagnosis means is very unreliable; however, the area of isolation should be included in the identification process, especially as old or new world.

In addition, classification by clinical symptoms has been misleading. Two L. b. panamensis isolates (111C and 111LN), have been diagnosed as causing visceral disease, while the visceral type is very often diagnosed as a cutaneous (WR285, WR317, WR341 and WR520). The most important misdiagnosis is present in the case of DCL, which in many patients shows as only simple cutaneous depending on the host reaction to the parasite.

Because of the discrepancies in clinical symptoms and country of isolation and the need, in some cases, of an early treatment (simple cutaneous being self-healing), identification by CAE is of importance as a rapid, simple, reproducible and accurate technique yielding permanent results.

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