UNIVERSIDAD DE GRANADA. INSTITUTO DE BIOTECNOLOGIA

FACULTAD DE CIENCIAS

CARACTERIZACION BIOQUIMICA Y MOLECULAR DE NUEVOS AISLADOS DE LOS GENEROS LEISHMANIA Y TRYPANOSOMA DE DIFERENTES PAISES: PERU, MEXICO Y ESPAÑA

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El presente trabajo de Investigación titulado: **Caracterización bioquímica y molecular de nuevos aislados de los géneros** *Leishmania* **y** *Trypanosoma* **de diferentes países; Perú, México y España,** ha sido realizado en el laboratorio del Departamento de Parasitología Molecular de la Facultad de Ciencias-Universidad de Granada, bajo la dirección y supervisión de:

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A Sara

A Jose

A mis padres

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INTRODUCCION.

INTRODUCCION.

Los Tripanosomátidos representan uno de los mayores problemas en casi todos los países del mundo, ya que son fuentes principales de morbilidad, mortalidad y perjuicio económico. Son responsables de importantes afecciones o enfermedades que afectan tanto a los seres humanos, animales, como a plantas.

Entre dichas enfermedades se encuentra la tripanosomiasis africana (Enfermedad del Sueño) considerada, después de la malaria, como una de las enfermedades más importantes de las que azotan al continente africano (Wang, 1995), y está causada por dos nosodemas de *Trypanosoma brucei*: el nosodema gambiano que predomina en el oeste de África y ocasiona una afección crónica; y el nosodema rhodesiano que en el este de África es causante de la afección aguda (Bales, 1991). Además del sufrimiento humano, el ganado, principalmente vacuno, se ve severamente afectado por *T. b. brucei*, que causa la tripanosomiasis bovina o "nagana".

La tripanosomiasis americana o enfermedad de Chagas, cuyo agente etiológico es el protozoo flagelado *Trypanosoma cruzi*, descubierto por Carlos Chagas en 1909. Comparada con enfermedades tropicales como la malaria y la leishmaniasis, la enfermedad de Chagas es considerada hoy en día como una gran carga socioeconómica en América tropical. Su importancia en salud pública radica en que es una enfermedad crónica que causa discapacidad (7 al 15% de los pacientes) y muerte.

T. cruzi se caracteriza por provocar una fase aguda después de la infección inicial y una fase crónica en la que se produce un desorden progresivo degenerativo de los sistemas cardiaco y gastrointestinal. Al

menos 18 millones de personas están infectadas por este parásito en países latino- americanos (OPS/OMS, 2003). La transmisión vectorial de *T. cruzi* está restringida al Nuevo Mundo y es llevada a cabo por varias especies de triatominos distribuidos en el continente americano entre los paralelos 42ºLN y 46ºLS, concretamente la región comprendida desde México hasta Argentina y Chile, que afecta principalmente a la población rural y peri-urbana, donde las viviendas construidas con adobe y carrizo, y el hacinamiento favorecen la presencia y proliferación del vector.

La información concerniente a la epidemiología de la enfermedad de Chagas en las diferentes áreas de Latinoamérica es muy variable. Como por ejemplo en Perú, donde se ha mostrado la existencia de 18 especies de triatominos. El vector principal del *T. cruzi* es *Triatoma infestans* en el Sur de Perú, mientras que, en la zona norte y nororiente habitan varias especies de triatominos implicados en la transmisión y, en algunos de ellos, se han encontrado de forma natural tripanosomátidos compatibles con *T. cruzi* y/o *Trypanosoma rangeli* (Abraham y col., 2002).

T. rangeli es un protozoo digenético, parásito de diversas especies de animales domésticos y silvestres, así como de humanos. Este parásito es patogénico para el vector (insectos triatominos del género *Rhodnius*) pero aparentemente inofensivo en humanos. Por ello, el tratamiento en personas infectadas con este parásito está considerado innecesario. Este flagelado está ampliamente diseminado en América Central y del Sur, muchas veces superponiendo su distribución geográfica con el agente etiológico de la enfermedad de Chagas, *T. cruzi*.

Sin embargo, *T. rangeli* induce una respuesta inmune humoral con aumento del nivel de anticuerpos que da reacción cruzada con *T. cruzi*

interfiriendo en el diagnóstico de la enfermedad de Chagas. Además, ambos son flagelados morfológicamente similares, aunque biológica y bioquímicamente son distintos. El principal problema es que, epidemiológicamente, comparten vectores del género *Rhodnius* y mamíferos reservorios, principalmente marsupiales. Por lo que, el aspecto primordial en el estudio del *T. rangeli* es la imperiosa necesidad de su correcta identificación y diferenciación con *T. cruzi* para poder proporcionar las medidas oportunas en la lucha frente a la enfermedad de Chagas (Cuba, 1998).

En México, el territorio que se encuentra a menos de 1800 m sobre el nivel del mar se considera como área endémica probable de la enfermedad de Chagas, ya que, a esa altura es donde se han encontrado triatominos infectados por el parásito. Pertenecientes al género *Triatoma* y a la especie *dimidiata*, pero estos insectos son también vectores de *T. rangeli* (Zavala-Velázquez, 2003).

Las leishmaniasis son otro grupo de importantes enfermedades producidas por miembros de la familia Trypanosomatidae y más concretamente, por especies del género *Leishmania.* Comprenden un conjunto de manifestaciones clínicas muy variables y están relacionadas en parte a la cepa del agente infeccioso, al medio ambiente y la respuesta inmune del hospedador. La infección puede localizarse en la piel y/o el tejido subcutáneo con o sin adenopatías regionales (leishmaniasis cutánea), en la mucosa oronasal (leishmaniasis mucocutánea) o afectar de forma generalizada al sistema mononuclear fagocítico (leishmaniasis visceral). Cada especie de Leishmania tiene una distribución geográfica específica y causa unos síndromes clínicos característicos. Entre ellas tienen en común el agente causal (alguna especie de *Leishmania*), el vector (insectos

dípteros hematófagos), el reservorio (vertebrados) y el parasitismo de las células del sistema fagocítico mononuclear (sobre todo macrófagos).

Entre las diferentes afecciones que producen encontramos la leishmaniasis visceral o Kala-azar, producida por *Leishmania Leishmania donovani* y *L. (L.) infantum*, en la cuenca del Mediterráneo, y *L. (L.) chagasi* (en el continente americano). Originan un grave problema de salud pública en el ámbito mundial, por su distribución geográfica, pues prácticamente todos los países intertropicales evidencian casos humanos. Aunque, de una forma mayoritaria se aprecia que en los países del Tercer Mundo los niveles culturales, los recursos económicos y sanitarios no permiten una buena determinación de su prevalencia, y obstaculizan los programas de control y lucha. Tampoco hay que olvidarse de las afecciones cutáneas y mucocutáneas que originan otras especies de este mismo género, de gran importancia en la salud pública (Murray y col., 2000).

En América, 20 especies de *Leishmania* del Nuevo Mundo se agrupan en 2 grupos taxonómicos, el subgénero *Leishmania Leishmania* y *Leishmania Viannia*. El subgénero *Viannia*, conocido como complejo *braziliensis*, incluye las especies *L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) panamensis* y *L. (V.) guyanensis*. Mientras que el subgénero *Leishmania* puede ser dividido en 2 complejos de especies: el complejo *mexicana* (*L.(L.) mexicana*, *L. (L.) amazonensis*, *L. (L.) garnhami*, *L. (L.) aristidesi* y *L. (L.) pifanoi*) y el complejo *donovani* (*L. (L.) chagasi*) (Laison y Saw, 1987).

La leishmaniasis cutánea y mucocutánea es una enfermedad de alta prevalencia en muchas áreas tropicales y subtropicales del mundo. Descrita

en 24 países de América, extendiéndose desde el sur de los Estados Unidos hasta el norte de Argentina.

Esta enfermedad constituye un grave problema de salud pública por los altos costos que representa a nivel psicológico, socio-cultural y económico. La leishmaniasis representa un serio obstáculo para el desarrollo socioeconómico de 88 países, de los que 72 están en vías de desarrollo, como Perú y México. Por lo cual, la Organización Mundial de la Salud ha reconocido a esta enfermedad como un problema de salud pública global. Se estima que unos 350 millones de personas pueden estar en riesgo de contraer la enfermedad y que unos 12 millones están infectados en todo el mundo. Según la OMS anualmente aparecen de 1,5 a 2 millones de casos nuevos aunque oficialmente solo se reconocen 600.000, se considera que 500.000 sufrirán leishmaniasis visceral y casi un millón y medio sufrirán leishmaniasis cutánea (OPS/OMS, 2003). Además, está considerada como una de las seis enfermedades tropicales de mayor importancia en términos de investigación para la búsqueda de nuevos métodos de prevención, diagnóstico y tratamiento.

Existen en todo el mundo cerca de 30 especies de leishmania que infectan a los animales, de las cuales 21 pueden infectar al hombre. En el Nuevo Mundo, son reconocidas un total de 20 especies del género *Leishmania*, llamadas leishmanias neotropicales de las cuales, 14 son capaces de infectar a humanos.

En Perú, la leishmaniasis constituye la segunda enfermedad endémica de tipo tropical y la tercera causa de morbilidad por enfermedades transmisibles después de la malaria y la tuberculosis. Habiéndose identificadas cinco especies: en la región amazónica son tres

las causantes de la leishmaniasis cutánea y mucocutánea, conocida en la región como leishmaniasis selvática o **Espundia**: *L. (L.) amazonensis, L. (V.) guyanensis* y *L. (V.) braziliensis*, siendo esta última la de mayor importancia en la zona. *L. (V.) lainsoni* localizada al este de los Andes y *L. (V.) peruviana* endémica de Perú y causante de la leishmaniasis cutánea andina (**Uta**) encontrándose en áreas entre los 600-3000 metros de altura y variando también en latitud, no encontrándose en la zona amazónica (Ampuero Vela, 2000).

La leishmaniasis cutánea andina "**Uta**" es una enfermedad predominantemente rural en Perú y reviste carácter endémico a nivel de la región neotrópica del continente americano. A pesar de su extensión, la enfermedad se centra en zonas bióticas muy bien definidas, con una densa vegetación donde conviven una elevada densidad de vectores (*Lutzomyia)*, y donde existen reservorios naturales que se convierten en fuentes de infección leishmaniósica para los insectos y las poblaciones susceptibles de dichas fases endémicas (Perez y col., 1994).

La confirmación parasitológica del diagnóstico es crítica porque el amplio espectro de síntomas puede ser causado por numerosos agentes etiológicos. Por otro lado, el tratamiento de la leishmaniasis es caro, largo y está asociado con graves efectos tóxicos.

En México, las especies reconocidas que producen la leishmaniasis cutánea y mucocutánea son del subgénero *Leishmania: L. (L.) mexicana* y del subgénero *Viannia*: *L. (V.) braziliensis*. Hasta 1991 sólo se habían notificado 5 casos de leishmaniasis visceral, y entre 1991 y 1993 se registraron 7 casos (OMS, 1996). La leishmaniasis cutánea afecta, al menos, a 17 estados. La distribución de *L.* (*L.*) *mexicana* es más amplia,

mientras que, *L. donovani chagasi* y *L.* (*V.*) *braziliensis* tienen distribuciones muy reducidas.

En Europa, la leishmaniasis cutánea y la visceral son endémicas de todos los países mediterráneos, entre los que se incluye España (con una seroprevalencia comprendida entre 1,7 y 48,4%). La mayoría de los casos clínicos contabilizados pertenecen a *L. (L.) infantum* (cepas tanto dermotrópicas como viscerotrópicas) (Bogdan y col., 2001). La leishmaniasis visceral producida por *L. (L.) infantum* es una zoonosis, la cual tiene al perro como reservorio. Pero en comparación a un ciclo zoonótico convencional, *L. (L.) infantum* puede en algunos casos seguir un ciclo antroponótico.

Hasta el momento no se disponen de fármacos idóneos ni para el tratamiento de la Leishmaniasis ni para la enfermedad de Chagas. Y los que se utilizan en la actualidad conllevan una serie de problemas entre los que incluyen una eficacia variable, un tratamiento largo no carente de toxicidad y la obligada administración parenteral para algunos de ellos.

Ciertas formas de leishmaniasis cutánea no necesitan tratamiento al ser autolimitadas; sin embargo, la leishmaniasis visceral (sintomática o no) y la mucocutánea sí lo precisan. Los fármacos de elección clásicamente han sido los antimoniales pentavalentes y la pentamidina. Frecuentemente aparece resistencia y/o mala respuesta de *Leishmania* sp. a estos compuestos.

La quimioterapia de elección frente a *T. cruzi* es el nifurtimox, el benznidazol, el allopurinol, y el melarsoprol todos ellos causan un amplio rango de efectos adversos (Maudlin y col., 2004).

PLAN DE TRABAJO.

La identificación y caracterización precisa de las especies es importante porque pueden requerir distintos regímenes de tratamiento. La caracterización de las especies, tanto de *Leishmania*, como de *Trypanosoma* es necesaria para alcanzar los objetivos epidemiológicos, así como para documentar la distribución de las especies y diseñar unas medidas de control apropiadas. (Harris y col., 1998).

Por todo esto, el objetivo de la presente memoria es la caracterización de diferentes cepas de *Leishmania* y *Trypanosoma* aisladas por nosotros en diferentes regiones de Perú, México y España.

La caracterización se ha realizado por diversas técnicas bioquímicas y moleculares:

1.- Aglutinación con lectinas, utilizándose las lectinas ConA de *Canavalia ensiformis*, VV de *Vicia villosa*, WGA de *Triticum vulgaris* y PNA de *Arachis hypogea*.

2.- Análisis isoenzimático por isoelectroenfoque en un PhastSystem, usando geles IEF de distinto pI (Pharmacia), utilizando diferentes sistemas enzimáticos: enzima málica (ME), glucosa 6-fosfato dehidrogenasa (G6PDH), isocitrato dehidrogenasa (IDH), malato dehidrogenasa (MDH), glucosa fosfato isomerasa (GPI), fosfoglucomutasa (PGM) y superóxido dismutasa (SOD).

3.- Análisis electroforético del ADN del kinetoplasto (ADNk) usando diferentes endonucleasas de restricción: Hae III, BamH I, Hinf I, EcoR I y MspI.

4.- Amplificación por PCR de determinadas secuencias muy conservadas en todas las especies.

5.- Resonancia magnética nuclear de protones.

Las cepas caracterizadas vienen reflejadas en la **tabla 1**.

Tabla 1

Origen y designación de los aislados de los géneros *Trypanosoma* y *Leishmania* de Perú, México y España y de las cepas de referencia utilizadas en este estudio.

Nuestro Código	Código Internacional	Origen Geográfico	Lugar de Aislamiento	Hospedador
TC	*T. cruzi* cepa Maracay,	Maracay (Venezuela)	Sangre	Humano
TY	*T. cruzi* cepa Y	Brazil	Sangre	Humano
TCL	*T. cruzi* cepa CL	Rio Grande do Sul (Brazil)	Heces	*Triatoma infestans*
TRa	*T. rangeli*	Dept. Cajamarca (Perú)	Gländ. Salivales	*Rhodnius ecuadoriensis*
TM 5		Yucatan (Mexico)	Heces	*Rhodnius prolixus*
TP 504		Dept. La Libertad (Perú)	Heces	*Panstrongylus chinai*
TP 702		Dept. Amazonas (Perú)	Heces	*Triatoma carrioni*
TP 704		Dept. Arequipa (Perú)	Heces	*Triatoma infestans*
TP 706		Dept. Amazonas (Perú)	Heces	*Triatoma herreri*
TRa 605		Dept. La Libertad (Perú)	Gländ. Salivales	*Rhodnius ecuadoriensis*
TRa 606		Dept. La Libertad (Perú)	Gländ. Salivales	*Rhodnius ecuadoriensis*
L.(L.) donovani	LCR-L 133 LRC, Jerusalem (Israel)	Begemder (Ethiopia)	LV	Humano
L. (V.) peruviana	MHOM/PE/84/LC26	Dept. La Libertad (Perú)	LC	Humano

Tabla 1. Continuación 1

Nuestro Código	Código Internacional	Origen Geográfico	Lugar de Aislamiento	Hospedador
L. (L.) infantum I	MCAN/ES/2001/UCM-10	Madrid (España)	LC	Perro
L. (L.) infantum II	MCAN/2000/UCM-1	Madrid (España)	LC	Perro
L.(*L.*) *amazonensis*	MHOM/BR/73/M1845	Brazil	LC	Humano
L. (*V.*) brazilensis	MHOM/ BR/75/M2904	Brazil	LMC	Humano
LP 1	“	Dept. La Libertad (Perú)	LC	Humano
LP 2	“	Dept. La Libertad (Perú)	LC	Humano
LP 3	“	Dept. La Libertad (Perú)	LC	Humano
LP 4	“	Dept. La Libertad (Perú)	LM	Humano
LP 5	“	Dept. Loreto (Perú)	LC	Humano
LP 6	“	Dept. Amazonas (Perú)	LC	Humano
LP 7	“	Dept. Ucayali (Perú)	LC	Humano
LP 8	“	Dept. Ucayali (Perú)	LC	Humano
LP 9	“	Dept. Huanuco (Perú)	LC	Humano
LP 10	“	Dept. Huanuco (Perú)	LC	Humano
LP 11	“	Dept. Huanuco (Perú)	LMC	Humano
LP 12	“	Dept. Loreto (Perú)	LC	Humano

Tabla 1. Continuación 2

Nuestro Código	Código Internacional	Origen Geográfico	Lugar de Aislamiento	Hospedador
LP 13	“	Dept. Loreto (Perú)	LC	Humano
LP 14	“	Dept. Loreto (Perú)	LC	Humano
LM 1	“	Dept. Campeche (México)	LC	Humano
LM 2	“	Dept. Campeche (México)	LC	Humano
LM 3	“	Dept. Campeche (México)	LC	Humano
LS 1	“	Madrid (España)	LC	Perro

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ORIGINAL PAPER

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Biochemical characterization of new strains of *Trypanosoma cruzi* and *T. rangeli* isolates from Peru and Mexico

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Abstract Seven trypanosome stocks isolated have been characterized by lectin agglutination, isoenzyme analysis, and the end products excreted. The stocks were isolated from different geographic areas—one from Mexico (TM5), and six from Peru, four of these isolated from different species of triatoma (TP504, TP702, TP704 and TP706), the other two isolated from the salivary glands of *Rhodnius ecuadorensis* (TRa605 and TRa606). Additionally, one strain of *Trypanosoma cruzi* isolated from a human case (strain TC-Maracay) and one strain of *T. rangeli* (TRa, Cajamarca-Peru strain), characterized and maintained in our laboratory, were used as reference strains. According to statistical study, the stocks were grouped into three clusters: (1) cluster I included the reference strain of *T. cruzi* (TC-Maracay); (2) cluster II was subdivided into two groups—subcluster IIA for the Mexican isolate (TM5) and subcluster IIB for the Peruvian ones, isolated from the salivary glands of *Rhodnius ecuadorensis* (TRa 605 and TRa 606) and the reference strain *T. rangeli* (TRa); these two new isolates were classified as *T. rangeli*; and (3) cluster III for the rest of the Peruvian isolates, which should be considered at least as a different strain from the *T. cruzi* strain Maracay. We show that the identification of *T. cruzi* and *T. rangeli* in mixed infections is readily achieved by biochemical methods. These findings identified three clusters of Mexican and Peruvian stocks that correlate with geographic origin, although assignment to a *T. cruzi* linage was not possible.

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Introduction

Chagas disease or American trypanosomiasis, caused by *Trypanosoma cruzi*, afflicts every country in South America (WHO 1983). This highly pleomorphic parasite has a complex life cycle involving a large number of triatomine insects and mammalian species. Isolation and study of *T. cruzi* populations from different origins demonstrated the presence of a large range of strains with distinct biological, biochemical and pharmacological characteristics (Rodríguez et al. 1998). This intriguing intraspecific variation has been extensively investigated by biological characterization. One widely used method is isoenzyme analysis, which has revealed different populations between the zymodeme and the transmission cycle (Miles et al. 1977, 1978; Ebert 1982; Schottelius and Muller 1984; Ebert 1985; Tibayrenc and Ayala 1988; Steindel et al. 1995). Also, lectins with different specificities for surface-membrane carbohydrate residues were used for taxonomic purposes (Miercio et al. 1980; Marinkelle et al. 1986). Recent studies of samples from various Latin American countries have shown that different epidemiological and biological characteristics are associated with the two different lineages of *T. cruzi*, that it is possible by isoenzyme typing and lectins to distinguish zymodeme I and II of *T. cruzi*, and that by lectins both inter- and intraspecific distinctions of *T. cruzi* and *T. rangeli* are given (Schottelius 1987; Fernándes et al. 1998). Thus, for a better understanding of the epidemiology and of the host–parasite relationship of *T. cruzi*, it is essential to identify strains of the parasite (Ebert 1982).

Trypanosoma rangeli, first described by Tejera (1920), is a protozoan parasite found in Central and South America, attacking a wide range of mammals (including humans), and carried by triatomine bugs. As this species

often appears together with *T. cruzi*, it is essential to characterize strains of both *T. rangeli* and *T. cruzi* for a better understanding of the interactions between the different subpopulations, this information being crucial in the Chaga disease clinic (Schottelius 1987; Steindel et al. 1991).

In a previous paper characterizing six trypanosomatids isolated from different geographical areas from South America using biochemical methods, we reported that several clusters could be distinguished; these strongly correlated with the geographical origin. Cluster I grouped isolates from Peru and the *T. cruzi* Maracay strain, while cluster II grouped the Brazilian isolates and another identified as *T. rangeli* (Cajamarca-Peru strain; Miralles et al. 2002). In the present study, we report the comparison of the results of a lectin-agglutination test, electrophoretic analysis of isoenzyme profiles and ^{1}H-NMR spectroscopy used to characterize other, new trypanosomatids isolated from different geographical areas in Mexico and Peru. Morphologically, all these have been considered members of the family Trypanosomatidae. The reference used from a human case was typified as the *T. cruzi* strain Maracay, and *T. rangeli* was characterized by Miralles et al. (2002).

Materials and methods

Parasite isolation and in vitro culture

The seven stocks have been recently isolated from different areas of Mexico and Peru: TM5 from *Rhodnius prolixus* in the Yucatan Peninsula of Mexico; the TP504 isolate of *Panstrongylus chinai* in the department of La Libertad in northern Peru; the TP702 isolate of *Triatoma carrioni* in the department of Amazonas in northeastern Peru; the TP704 isolate of *T. infestans* in the department of Arequipa in southern Peru; the TP706 isolate of *Triatoma herreri* from the department of Amazonas; and the TRa605 and TRa606 isolates, both from the salivary glands of two *Rhodnius ecuadoriensis* in the department of La Libertad, Peru. For comparison, we also included *T. cruzi* (TC) Maracay, originally isolated from a clinical case in the Malariological Division of Environmental Health of Maracay (Venezuela), and *T. rangeli* (TRa, Cajamarca-Peru strain), isolated from the salivary glands of *Rhodnius ecuadoriensis* (Miralles et al. 2002) and maintained in our laboratory by routine procedures (Sánchez-Moreno et al. 1995).

After isolation, the new flagellates were transferred to the biphasic culture medium Nicolle Nocloe Novi and the MacNeal medium, supplemented with minimal essential medium, and 20% inactivated fetal bovine serum. The isolates were cloned and cultured in vitro using Grace's medium (Sigma; Sánchez-Moreno et al. 1995). The parasites were inoculated at a density of 1.6×10^{6} into 5 ml of medium in 25-ml Falcon flasks, and cultured at 28°C. Aliquots were taken every 24 h for 9 days, and the number of parasites was counted in a Neubauer hemocytometer. On the last day, the cells were removed by centrifugation at 600 *g* for 10 min, and the pH of the parasite-free supernatants was measured.

Lectin-agglutination test

Lectins from *Canavalia ensiformis* (Con A; from Sigma Chemical Co. St. Louis, Mo, USA), *Vicia villosa* (VV; from Sigma Chemical Co. St. Louis, Mo, USA), *Triticum vulgaris*, a wheat-germ agglutinim (WGA; from Sigma Chemical Co. St. Louis, Mo, USA), and *Arachis hypogaea* (PNA; from Sigma Chemical Co. St. Louis, Mo, USA) were used. Parasites (in the initial growth phase) were washed three times by centrifugation (600 *g* 10 min, 4°C), and resuspended in phosphate-buffered saline, pH 7.4, containing 0.5% inactivated fetal bovine serum, at a concentration of 10^{8} parasites/ml; autoagglutinations were not observed. Tests were performed in duplicate in 96-well trays (Flow Laboratories), in which 50 µl of parasite suspension was incubated with equal volumes of lectins at different concentrations for 1 h at room temperature, for final lectin concentrations of 1, 10, 20, 50, 100, 150, 500, 750, and 1,000 µg/ml (Andrade and Saraiva 1999). Agglutination was determined by microscope observation according to Zubiaur and Alonso (1985). The agglutination tests were controlled by inhibition tests using the following sugars: *Canavalia ensiformis* plus 0.1 M α-D-glucosyl and 2% D-manose; *Vicia villosa* plus 0.1 M N-acetyl-D-galactosamine; and *Triticum vulgaris* plus 0.1 M N-acetyl-D-glucosamine. In an additional reaction, the parasites were incubated in a volume of phosphate-buffered saline, pH 7.4, equal to the volume of lectins to control for an eventual tendency of the parasites for autoagglutination (Schottelius 1982).

Isoenzyme characterization

Crude homogenates were obtained from 250 ml of culture medium containing 2×10^{7} cells/ml. Cells were harvested by centrifugation at 1,500 *g* for 10 min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme-stabilizer solution containing 2 mM dithiothreitol, 2 mM E-aminocaproic acid, and 2 mM EDTA (Ben Abderrazak et al. 1993). The samples were frozen at −80°C for 15 min, and thawed at 25°C. After several freezing–thawing cycles, cell lysates were centrifuged at 8,000 *g* for 20 min at 4°C, and the supernatants stored in liquid nitrogen until used. The protein concentration was determined using the Bradford method, and storage was at a final concentration of 1 mg/ml of protein. The enzymes were separated by isoelectric focusing in a PhastSystem apparatus, using Phast-gel IEF 3-9 (Pharmacia-LKB). The following enzymes were tested: glucose phosphate isomerase (GPI; EC 5.3.1.9), isocitrate dehydrogenase (IDH; EC 1.1.1.42), malate dehydrogenase (MDH; EC 1.1.1.37),

malic enzyme (ME; EC 1.1.1.40), phosphoglucomutase (PGM; EC 2.7.5.1), and superoxide dismutase (SOD; EC 1.15.1.1). The staining procedures are described in Férnandez-Ramos et al. (1999).

^{1}H-NIMR spectroscopy and metabolite identification

For the spectroscopic assessments, 5 ml of a 5-day-old culture of each isolate in Grace's medium was centrifuged at 1,500 *g* for 10 min at 4°C. The pellet was discarded, and the parasite-free supernatant was stored at −20°C until used. The ^{1}H-NMR spectra were determined by the method described in an earlier study; the chemical displacements used to identify the metabolites were consistent with those of Sánchez-Moreno et al. (1992).

Statistical study

The statistical methods were based on individual hierarchical cluster analysis, selecting the Euclidean distance to the square as the basis for measuring the associations between individuals. The Euclidean distance was calculated by the following grouping procedures: simple linkage (R_k 0.7355), average linkage among groups (R_k 0.7518), average linkage (R_k 0.7726), the centroid method (R_k 0.7586), the median method (R_k 0.7204), and the Ward method (R_k 0.7570). The cophenetic coefficient (R_k) measures the degree of distortion between relationships, means in terms of original distances between individuals, and those existing at the end of the analysis. The method with the highest cophenetic correlation was chosen as the optimal one. In the selection procedure, the average linkage between groups was considered, using the coefficient R_k of Rand, which is an index of the similarity between classifications. This analysis was made with the StatGraphics program, version 5.0.

Results

In general, all the isolates grew satisfactorily in Grace's medium supplemented with 10% IFCS (Fig. 1), reaching 7×10^6–1.8×10^7 cells/ml, depending on the isolate. In all cases, the initial concentration was 1.6×10^6 cells/ml, and growth remained exponential during the first few days of culture, becoming stationary on day 5. Figure 1 presents only the growth curves of the isolates TM5 and TP704 as well as the two reference strains, TC-Maracay and Tra. The isolates TP702 and TP706 show a growth curve similar to that of TP704, while the curve of the isolates from the salivary glands of *R. ecuadoriensis* (TRa 605 and TRa 606) resembles that of Tra, and the curve of TP504 is similar to that of TC-Maracay (data not shown).The nine isolates showed a clear agglutination

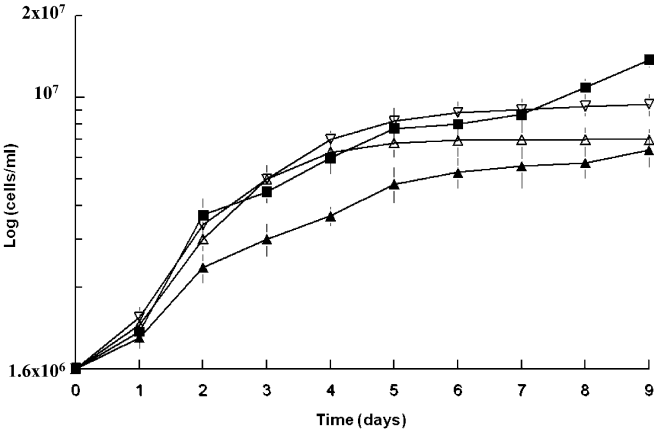

Fig. 1 Growth curves of isolates in Latin America in Grace's culture medium: *open triangles* TC, *filled triangles* TM5, *filled squares* TP704, *inverted triangles* TRa (±SD of the mean of three different experiments)

with the lectin Con A at 1 μg/ml (Table 1), although the degree of agglutination varied according to the isolate. The lectin *Vicia villosa* (VV), at all dosages assayed, agglutinated only the isolates TRa, TRa605, and TRa606 at concentrations of 20, 20, and 5 μg/ml, respectively. The wheat-germ lectin (WGA) displayed a heterogeneous agglutination pattern within the different isolates; TRa605 was agglutinated at the minimum concentration (1 μg/ml); TC, TRa, and TRa606 were agglutinated at a concentration of 5 μg/ml; and the isolates TM5, TP504, TP702, TP704, and TP706 were agglutinated at 20 μg/ml. PNA lectin agglutinated with all the isolates, except with the reference strain from Venezuela. The strong agglutination of the strain from Mexico (TM5) was noteworthy for this lectin. Cell agglutination by lectins was specific, as no spontaneous clustering of cells occurred in the absence of lectins, or in the presence of the specific saccharide inhibitors.

The isoenzymatic pattern of some of the isolates studied appears to reveal variations in the number of bands and their isoelectric points (Fig. 2). In general, the *T. cruzi* strain Maracay (TC) differs from the other isolates in the six systems studied (Fig. 2, line 1). The isoenzymatic pattern for the enzyme GPI (Fig. 2A) is the same as that for all the other isolates. The profile of IDH appears in Fig. 2B, reflecting in general wide variability in the number and mobility of the bands, although all the new isolates presented a band in common with the only band shown by TRa (Fig. 2B, line 9). According to the isoenzymatic profile of MDH, all the isolates shared a common band having an isoelectric point close to 9, although the isolates TP702, TP704, and TP706 (Fig. 2C, lines 4–6) presented a second band with an isoelectric point similar to the second band corresponding to TC (Fig. 2C, line 1). For the ME enzyme, an isoenzymatic profile (three bands) proved very similar in all the isolates, except for TP702 (Fig. 2D, line 4) with a single band that it shares with the rest of the isolates,

Table 1 Agglutination activities of lectins for nine *T. cruzi* isolates of different origins

Trypanosomatids isolates, origin			Minimal concentration of lectins required for agglutination[a]								Cluster
			Concavalina ensiformis ConA		*Vicia villosa* VV		*Triticum vulgaris* WGA		*Arachis hypogaea* PNA		
			(μg/ml)		(μg/ml)		(μg/ml)		(μg/ml)		
T. cruzi											
TC (reference strain)	Venezuela	Human	1	(+)		(−)	5	(+)	20	(−)	I
TM5	Yucatan, Mexico	Bug	1	(+)		(−)	20	(+)	40	(+++)	IIA
TP504	Northern Peru	Bug	1	(++)		(−)	20	(+)	40	(+)	III
TP702	Northeastern Peru	Bug	1	(++)		(−)	20	(+)	40	(+)	III
TP704	Southern Peru	Bug	1	(+)		(−)	20	(+)	40	(+)	III
TP706	Amazonas, Peru	Bug	1	(++)		(−)	20	(+)	40	(+)	III
T. rangeli											
TRa605	La Libertad, Peru	Bug	1	(+)	5	(+)	5	(+)	10	(+)	IIB
TRa606	La Libertad, Peru	Bug	1	(+)	20	(+)	1	(+)	10	(+)	IIB
TRa (reference strain)	Cajamarca, Peru	Bug	1	(+)	20	(+)	5	(+)	10	(+)	IIB

[a]Agglutination was scored from − (no agglutination at 1,000 μg/ml) to + (25%), ++ (50%), and +++ (virtually complete agglutination); average of three experiments

and TP706 with two bands (Fig. 2D, line 6). In the profile for PGM (Fig. 2E), it was found that all the isolates shared one band, although the Peruvian isolates TP504, TP702, TP704, and TP706 (Fig. 2E, lines 3–6) shared a second band with a different isoelectric point. All the isolates presented four similar bands for the

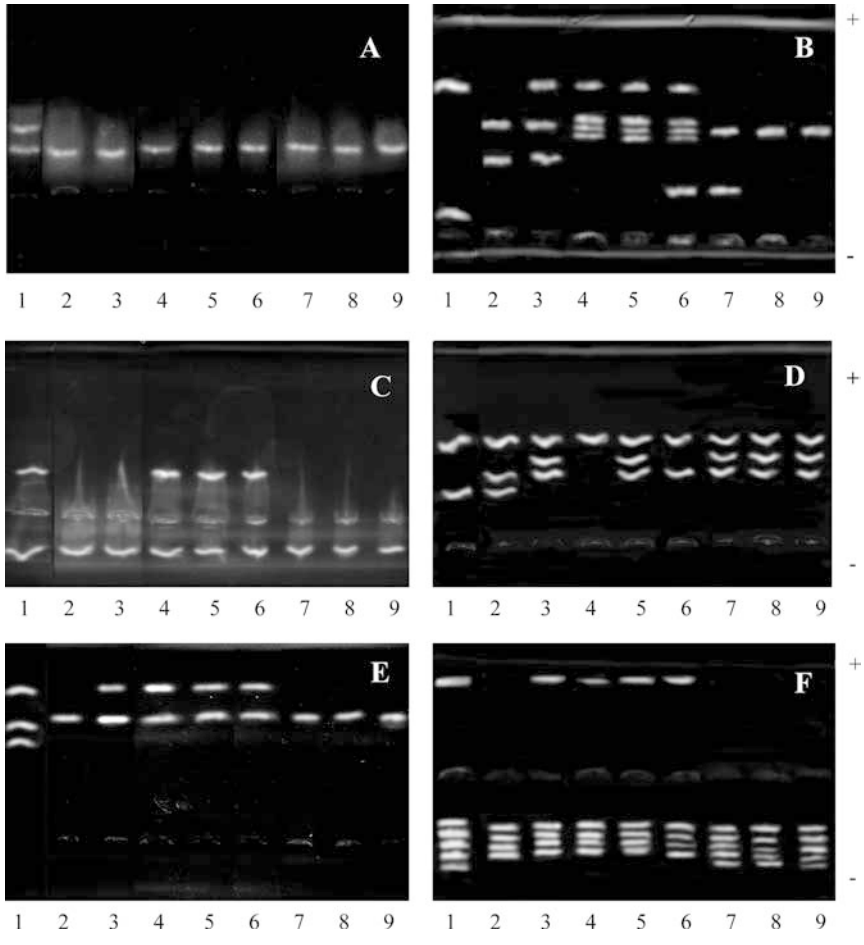

Fig. 2A–F Isoenzyme profiles of *1 T. cruzi*, Maracay strain, *2* isolate TM5, *3* isolate TP504, *4* isolate TP702, *5* isolate TP704, *6* isolate TP706, *7* isolate TRa606, *8* isolate TRa605, and *9* isolate TRa. Enzymes are **A** glucose phosphate isomerase (GPI), **B** isocitrate dehydrogenase (IDH), **C** malate dehydrogenase (MDH), **D** malic enzyme (ME), **E** phosphoglucomutase (PGM), and **F** superoxide dismutase (SOD)

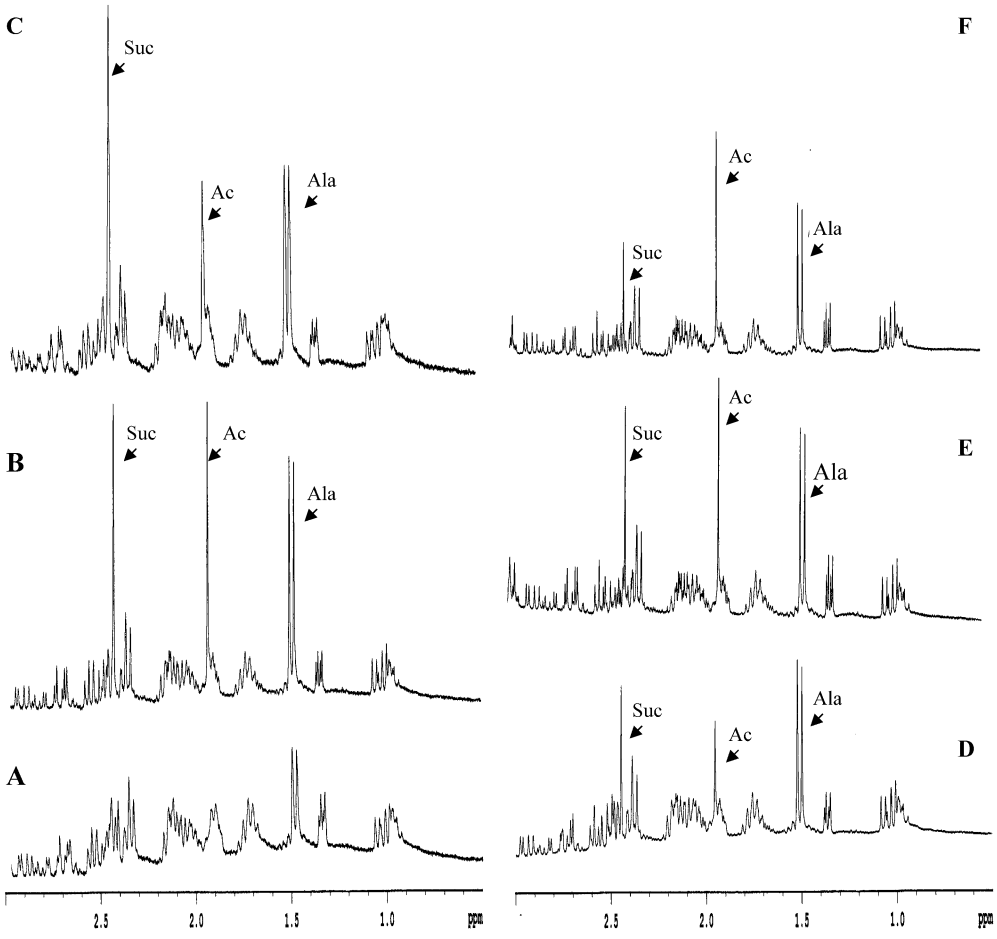

Fig. 3A–F ^{1}H-NMR spectra of the isolates culture medium. **A** Fresh culture medium before inoculation of cells; **B** spectra obtained for the cell-free culture media of the *T. cruzi* strain Maracay; **C** isolate TP704; **D** isolate TRa; **E** isolate Tra606; **F** isolate TM5. *Suc* Succinate, *Ac* acetate, *Ala* alanine

enzyme SOD (Fig. 2F); the isolates TRa606, TRa605, and the reference strain TRa also presented a fifth band, with an isoelectric point of close to 9, which they shared with the reference strain TC (Fig. 2F, lines 7–9, and line 1). In addition, this reference strain shared a band having an isoelectric point of 3.6 with the isolates TP504, TP702, TP704, and TP706 (Fig. 2F, lines 3–6, and line 1).

To identify the main metabolites excreted into the medium, we used ^{1}H-MNR spectroscopy to analyze the composition of the medium in which the different isolates had been grown for 5 days, as well as the cell-free medium used as control (Fig. 3). The additional peaks visible in the spectra corresponding to the media used to grow flagellates, in comparison with the control spectrum (Fig. 3A), indicated excreted metabolites. The ^{1}H MNR study indicated that for all the isolates the main metabolites excreted were succinate (Suc, 2.3 ppm), acetate (Ac, 1.8 ppm), and alanine (Ala, 1.4 ppm). Quantitative differences appeared between isolates; for example, for TP704, the main metabolite was succinate (Fig. 3C); acetate proved to be the main metabolite for TC, TRa 606, and TM5 (Fig. 3B, E and F, respectively); and alanine was the main metabolite for the isolates TRa (Fig. 3D) as well as TRa605, TP504, TP702, and TP706 (data not shown).

Discussion

The results of the lectin-agglutination test showed that the composition of carbohydrates of the membranes of the trypanosomes varied from one isolate to another. The nine isolates agglutinated with the lectin Con A, indicating that all had molecules of α-D-glucose and α-D-mannose in the composition of their membranes, although in the isolates TP504, TP702, and TP706 these molecules were more numerous than in the others, or presented a different arrangement that permitted agglutination. The lectin from *Vicia villosa* agglutinated only with trypanosomes isolated from the salivary glands of *R. ecuadoriensis* (TRa605 and TRa606) and the reference strain TRa, indicating that *N*-acetyl-galactosamine was among the sugars of its membrane, in addition to presenting a strongly marked agglutination at a very low concentration, this being evidence of an abundance of this sugar. These data agree with those of other authors (Acosta et al. 1991), who determined that VV lectin only selectively reacts with *T. rangeli* strains. The wheat-germ lectin (WGA) displayed a heterogeneous agglutination pattern within the different isolates, and thus the presence of *N*-acetyl-D-glucosamine residues among the surface sugars was highly variable.

PNA lectin clearly separates the TC reference with regard to the rest of the isolates from Peru and Mexico.

Different authors have found that strains from Venezuela and Brazil, including type 2, were PNA types, as they agglutinated with *Arachis hypogaea* lectin but not with WGA lectin (Vivas et al. 1979, Schottelius 1982), as occurred with our reference strain TC. The Mexican isolate (TM5) presented a strong agglutination with PNA lectin, which distinguishes the Peruvian isolates, demonstrating that neuraminic acids exist in abundance on the surface of the epimastigote from these isolates. According to statistical analysis by the group average method, the Euclidean of the lectin-agglutination test grouped the different isolates into three clusters: the first cluster was formed by the reference TC strain; the second by TM5, TP504, TP702, TP704, and TP706; and the third by TRa, TRa605, and TRa606 (data not shown).

The isoenzyme patterns of the three isolates from the salivary glands of *R. ecuadoriencis* are more homogeneous for the six enzymatic systems tested. The TC strain from Maracay showed differences in the loci of the six enzymatic systems with respect to the rest of the isolates from Peru and Mexico. The Peruvian isolates (TP702, TP704, and TP706) showed great similarity in the enzymatic loci GPI, MDH, ME, PGM, and SOD. Comparing our results with those of Ebert (1982), we find a strong coincidence in the profiles of some of the enzymes assayed (PGM, MDH, and ME), implying that these Peruvian isolates may be correlated to the *T. cruzi* isolates included within the zimodene Z1 by this author.

The statistical study of these results grouped the nine isolates into three clusters: the first includes the reference TC strain; the second includes the isolate from Mexico (TM5), the Peruvian isolates (TP504, TRa605, and TRa606), together with the strain isolated from *R. ecuadoriensis* of Peru and used as the reference strain of *T. rangeli* (TRa); and the third includes the rest of the Peruvian isolates (TP702, TP704, and TP706).

The members of the family Trypanosomatidae are incapable of completely degrading carbohydrates, even in the presence of oxygen, producing CO_2 and dicarboxylic acids (Sánchez-Moreno et al. 1995). In addition, the relative proportion of these end products varies among subspecies of *T. cruzi* (Urbina et al. 1993).

By ^{1}H-MNR spectroscopy, we identified the main metabolites excreted into the culture medium by the isolates. The analysis indicated that for all the isolates, the main metabolites excreted were succinate, acetate, and alanine, the proportion varying according to the isolate. Several authors have identified these same metabolites in other *T. cruzi* strains, such as the strain Tulahuen (Cazzulo 1994), strain Y (Rogerson and Gutteridge 1980), and the Bolivia strain of our group (Penin et al. 1998). Furthermore, other *T. cruzi* isolates from Brazil, characterized in a previous study, excreted other metabolites (Miralles et al. 2002). The differences found when the seven isolates were compared with the reference strains indicated changes in the metabolic strategies of the trypanosomes in degrading energy substrates, and showed that certain energy pathways are more developed in some strains than in others (Miralles et al. 2002).

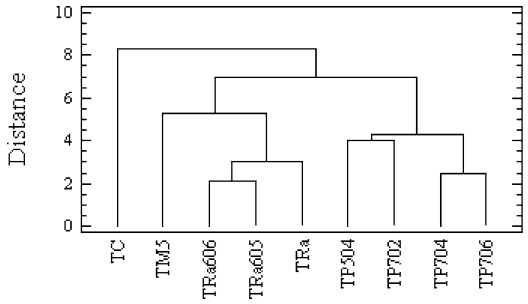

Fig. 4 Dendrogram based on individual hierarchical cluster analysis (program Stat-Graphics version 5.0)

The statistical analysis of all the results together (Fig. 4) provides a more real phylogenetic approach, and in this way the isolates were grouped into three clusters. Cluster I is the Maracay *T. cruzi* strain, which clearly differentiates the rest of the isolates. Cluster II can be divided into subcluster IIA, including the strain isolated from *R. prolixus* in the Yucatan Peninsula in Mexico, and subcluster IIB, including the isolates from the salivary glands of *R. ecuadoriensis* from the department of Libertad in Peru, together with the reference strain *T. rangeli*. This indicates that these isolates (TRa605 and TRa606) should be considered as belonging to the species *T. rangeli*. In support of this hypothesis, *T. cruzi* and *T. rangeli* differ in the isolation site of the flagellate, which in this case is *T. rangeli*, almost invariably in salivary glands (Grisard et al. 1999).

The third cluster includes the other four isolates from Peru (TP504, TP702, TP704, and TP706). Being phylogenetically distant from the Maracay strain of *T. cruzi*, these should be considered as belonging to another strain, although phylogenetically they have been recently separated from the TRa strains. The biochemical differences found suggest that the *T. cruzi* isolates are markedly heterogeneous, even when they come from the same host species and the same geographical region (Moreno et al. 2002). According to Tibayrenc et al. (1986), groups of clones that are biochemically similar in terms of their biological properties can be found. On the other hand, different clones with the same origin adapted to jungle, peridomestic, and domestic cycles would have conserved biochemical/enzymatic properties but would show different biological behavior.

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Identification and biochemical characterization of *Leishmania* strains isolated in Peru, Mexico, and Spain

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Abstract

Eight *Leishmania* promastigotes were isolated from different geographical areas: three (LP1, LP2, and LP3) from the provincial department La Libertad and the fourth (LP4) from the department of Cajamarca (northern Peru); another three (LM1, LM2, and LM3) in the province of Campeche (Mexico); and the last (LS1) from a clinical case of a dog in Madrid (Spain). The isolates were characterized by carbohydrate cell-surface residues using agglutinations with four purified lectins, by isoenzyme analysis using different isoenzymes, by analysis of kinetoplast DNA (kDNA) restriction fragment length polymorphism using four different restriction endonucleases and by the final metabolite patterns after in vitro culture. These isolates were compared with four reference strains and typified as: *Leishmania* (*Leishmania*) *donovani*, two strains of *L.* (*L.*) *infantum*, and one species of *L.* (*Viania*) *peruviana*. According to our results and the statistical study, the Peruvian isolates represent three different strains: one would be *L.* (*V.*) *peruviana*, another the strain isolated in Cajamarca (LP4) and the third would include the three strains from the department of La Libertad (LP1, LP2, and LP3), these latter three isolates being phylogenetically closer to the reference strain *L.* (*L.*) *donovani*. Meanwhile, the three isolates from Mexico form a group with close phylogenetic relationships to each other. The isolate from Spain belongs to the species *L.* (*L.*) *infantum*. Thus, a close correlation was drawn between the identity of each strain and its geographical origin.

Index Descriptors and Abbreviations: *Leishmania* species; In vitro culture; Lectin agglutination: Con A, lectin from *Canavalia ensiformis*; VV, lectin from *Vicia villosa*; WGA, lectin from *Triticum vulgaris*; PNA, lectin from *Arachis hypogae*; Isoenzyme electrophoresis: ME, malic enzyme [EC 1.1.1.40]; MDH, malate dehydrogenase [EC 1.1.1.37]; IDH, isocitrate dehydrogenase [EC 1.1.1.42]; GPI, glucose phosphate isomerase [EC 5.3.1.9]; PGM, phosphoglucomutase [EC 2.7.5.1]; SOD, superoxide dismutase [EC 1.15.1.1]; kDNA restriction pattern: kinetoplast deoxyribonucleic acid; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; MEM, minimal essential medium; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; DNA, deoxyribonucleic acid

1. Introduction

Human leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, constitutes a serious public health problem in several countries, according to the World Health Organization (W.H.O., 1997). In human hosts, the clinical profile of different *Leishmania* species can vary from a single cutaneous lesion, which may undergo spontaneous cure, to mucocutaneous lesions that can become grossly disfiguring. Severe diffuse cutaneous lesions, that is, extremely difficult to treat, can also occur. Moreover, the disease can evolve to visceral forms that are lethal in most cases (Ferreira et al., 2003).

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Clinicians are confronted with steadily higher numbers of leishmaniasis patients not only in countries where the disease is endemic but also in countries where these parasites are not endemic. This increased incidence is partly due to geographical expansion of the disease, changing patterns of international travel and population migration, non-immune people moving into endemic regions, or infected people moving into non-endemic regions (Desjeux, 2001).

Leishmania species are morphologically very similar and species identification is possible using standard biochemical methods (lectin agglutination, isoenzyme analysis, analysis of kDNA restriction fragment using different restriction endonuclease, etc.). As has been demonstrated in many works where these techniques have been satisfactorily used to characterize *Leishmania* isolates (Andrade and Saraiva, 1999; Belhadj et al., 2003; Sampali et al., 2003; Shamsuzzaman et al., 2000), the ability to distinguish between *Leishmania* species is crucial when prescribing treatment as well as when determining possible control measures in epidemiological studies. Frequently, *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. However, geographical origin is an inadequate criterion in non-endemic areas, as well as endemic regions where multiple species of *Leishmania* may co-exist. Identification of the infecting species based on clinical symptoms can be problematic, since several species cause both cutaneous and mucocutaneous disease, while others cause visceral and cutaneous disease (Schönian et al., 2003).

In the present work, we characterized eight *Leishmania* isolates from different areas of Latin America (Peru and Mexico) as well as from the Mediterranean region (Spain), using interaction of the parasites with lectins together with electrophoretic analysis of their isoenzyme profiles and analysis of kDNA restriction fragment. Morphologically, all these have been considered to be members of the genus *Leishmania*. For comparison, we used four isolates from human cases characterized as: *L.* (*L.*) *donovani*, two strains belonging to *Leishmania* (*L.*) *infantum* and another characterized as *Leishmania* (*Viania*) *peruviana*. In addition, we made a comparative study of the major end-products excreted into the culture medium by the parasites.

2. Materials and methods

2.1. Parasite isolation and in vitro culture

The eight *Leishmania* were isolated from different areas of Peru, Mexico, and Spain. Three of these isolates were from cutaneous cases in the central zone (LP1 and LP2) and north-eastern part (LP3) of the department of La Libertad (Peru); a fourth isolate (LP4) from a mucosal lesion was isolated in the zone of Cajamarca, in north-eastern Peru (near the border of Ecuador and Colombia). Three isolates (LM1, LM2, and LM3) were from cutaneous lesions in Campeche (Mexico) during the period 2000–2002. The final isolate (LS1), from the area of Madrid (Spain), was taken from a dog. For comparison, we also included: *L.* (*L.*) *donovani* (LCR-L 133, *Leishmania* Reference Center Jerusalem, Israel) isolated in a human case of kala-azar in Begemder (Ethiopia); *L.* (*V.*) *peruviana* (MHOM/PE/84/LC26) and two strains of *L.* (*L.*) *infantum*: I and II, characterized as MCAN/ES/2001/UCM-10 and MCAN/2000/UCM-1 isolated in Spain, respectively. (The reference strains have been maintained for several years in our laboratory by successive passes in cultures of NNN medium modified with a liquid phase of minimal essential medium (MEM) plus 10% inactivated foetal bovine serum kept in an air atmosphere at 28 °C. To maintain infectivity, a subculture was approximately every two weeks and at least every six months, was inoculated in the Syrian golden hamster, *Mesocricetus auratus*, and parasites are isolated from the spleen 30 to 45 days post-inoculation.

The isolates from hamsters were cloned and cultured in vitro as previously described (Sánchez-Moreno et al., 1995). Different monophasic cultures were tested: RPMI-1640, MTL, and TC medium (Gibco, Karlsruhe, Germany) and MEM (Sigma, St. Louis, MO).

The epimastigote forms of *Trypanosoma cruzi* strain Maracay were cultured in vitro using Grace's medium (Sigma) (Sánchez-Moreno et al., 1995).

2.2. Lectin-agglutination test

Lectins from *Canavalia ensiformis* (Con A), *Vicia villosa* (VV), *Triticum vulgaris*, a wheat-germ agglutination (WGA), and *Arachis hypogaea* (PNA) were used (Sigma, St. Louis, MO). Parasites were washed three times with phosphate-buffered saline, pH 7.4, and collected by centrifugation ($600g \times 10$ min, 4 °C), and resuspended in phosphate-buffered saline, pH 7.4, containing 0.5% inactivated foetal bovine serum. Tests were performed in duplicate in 96-well plates (Flow Laboratories). The parasite suspension (50 μl) was incubated with equal volumes of lectins at different concentrations for 1 h at room temperature (final lectin concentrations of 1, 10, 20, 50, 100, 150, 500, 750, and 1000 μg/ml). Agglutination was determined by microscopic observation according to (Zubiaur and Alonso, 1985). Controls for specific agglutination were performed using 0.1 M α-D-glucosyl for Con A, 0.1 M *N*-acetyl-D-galactosamine for VV, 0.1 M β-galactose for PNA, and 0.1 M *N*-acetyl-D-glucosamine for WGA.

2.3. Isoenzyme characterization

Crude homogenates were obtained from 250 ml of culture medium containing 2×10^7 cells/ml. Cells were harvested by centrifugation at $1500g \times 10$ min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme stabilizer solution containing 2 mM dithiothreitol, 2 mM *E*-aminocaproic acid, and 2 mM EDTA (Fernandez-Ramos et al., 1999). The samples were frozen at −80 °C for 15 min and thawed at 25 °C. After several freezing–thawing cycles, cell lysates were centrifuged at

8000g × 20 min at 4 °C, and the supernatants were stored in liquid nitrogen until used. The protein concentration was determined using the Bradford method and stored at a last concentration of 1 mg/ml of protein.

The enzymes were separated by isoelectric focusing in a PhastSystem apparatus, using Phast-gel IEF 3-9 (Pharmacia, Freiburg, Germany).

The following enzymes were tested: malic enzyme (ME), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), and superoxide dismutase (SOD). The staining procedures are described in Fernandez-Ramos et al. (1999).

2.4. Kinetoplast-DNA isolation, restriction enzyme digestion, and electrophoresis analysis

Promastigotes were collected by centrifugation of 300 ml of culture medium, after about 5 days. When their concentrations had reached about 2×10^7 cells/ml. They were washed twice in 50 ml of 0.15 M NaCl, 0.015 M sodium citrate and once with SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). Kinetoplast DNA was obtained according to the procedure described by Gonçalves et al. (1984).

The kDNA extracts (3 μg/μl) were digested to completion with restriction endonucleases (*Hae*III, *Bam*HI, *Hin*fI, and *Eco*RI) according to the manufacturer's prescribed buffer conditions (Boehringer–Ingelheim, Barcelona, Spain). The digestion products were electrophoresed in 1.5% agarose slab gels as described (Riou and Yot, 1977) and the fragment sizes were estimated by comparing their mobilities with those of a 100-bp DNA ladder (Gibco–BRL, Gaithersburg, USA). The gels were stained with ethidium bromide (10 μg/ml for 10 min) and photographed under UV light with a Polaroid camera (665 film).

2.5. ^1H NMR spectroscopy and metabolite identification

For the spectroscopic studies, 5 ml of a 5-day-old culture of each isolate in MEM was centrifuged at 1500g for 10 min at 4 °C. The pellet was discarded, and the parasite-free supernatant was stored at −20 °C until used.

The ^{1}H NMR spectra were determined according to a previous described method (Fernandez-Ramos et al., 1999). The chemical displacements used to identify the respective metabolites were consistent with those of Sánchez-Moreno et al. (1995).

2.6. Statistical study

The statistical methods were based on individual hierarchical cluster analysis, selecting the Euclidean distance to the square as the basis for measuring the associations between individuals. The Euclidean distance was calculated by the following grouping procedures of: simple linkage (R_k 0.7355), average linkage among groups (R_k 0.7518), average linkage (R_k 0.7726), centroid method (R_k 0.7586), median method (R_k 0.7204), and the Ward method (R_k 0.7570). The cophenetic coefficient (R_k) measures the degree of distortion between relationships, means in terms of original distances between individuals and those existing at the end of the analysis. The one with the highest cophenetic correlation was chosen as the optimal method.

In the selection procedures the average linkage between groups was considered, using the coefficient R_k of R and, which is an index of the similarity between classifications. This analysis was made with the StatGraphics program, version 5.0.

3. Results

Leishmania isolates were cultured in vitro, assaying different liquid media. In general, all the isolates grew satisfactorily in MEM (Fig. 1), reaching cell densities on the order of 6.5×10^6–1×10^7 cells/ml, depending on the isolate.

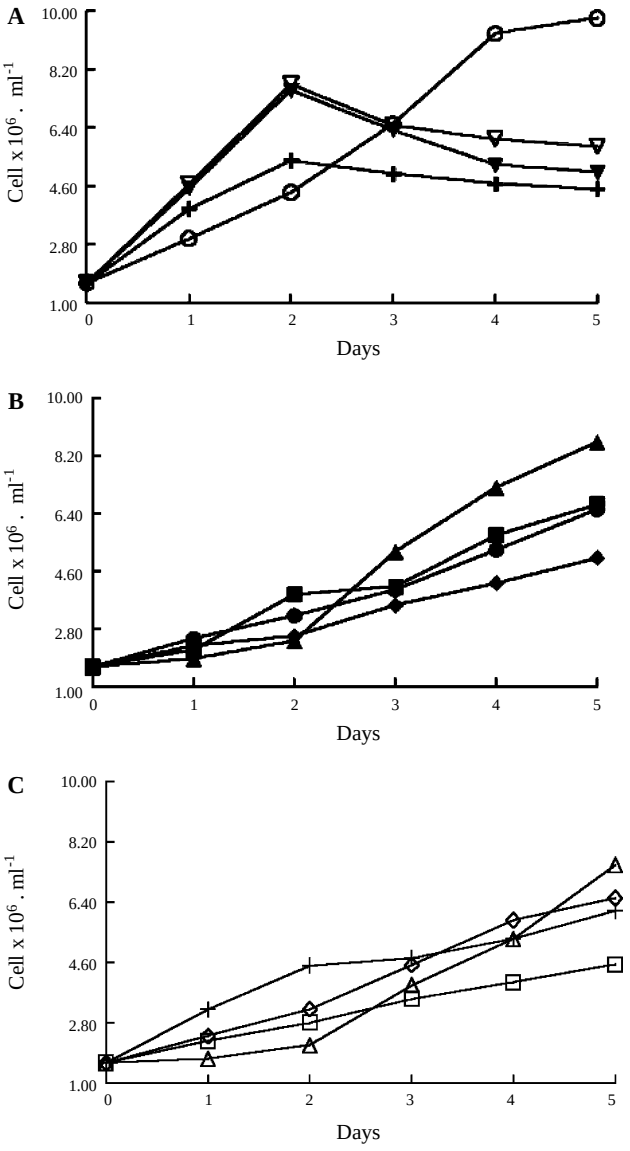

Fig. 1. Growth curves of *Leishmania* isolates in Latin America and Spain. (A) *L.* (*L.*) *donovani* (○), *L.* (*L.*) *infantum* I (▼), *L.* (*L.*) *infantum* II (▽), and LS1 (+). (B) *L.* (*V.*) *peruviana* (▲), LP1 (◆), LP2 (■), and LP3 (●). (C) LP4 (△), LM1 (+), LM2 (◇), and LM3 (□).

Table 1 presents the results for lectin-agglutination tests. All the isolates showed agglutination with the lectins Con A, but with different degrees of agglutination and minimum concentrations required to agglutinate. The reference strain *L.* (*V.*) *peruviana* weakly agglutinated at a concentration of 1 μg/ml; the three isolates from Spain (*L.* (*L.*) *infantum* I and II and LS1) agglutinated at 5 μg/ml, while the rest of the isolates and *L.* (*L.*) *donovani* needed a greater concentration of lectins (20 μg/ml), although for the isolates from Peru (LP1, LP2, and LP3) and Mexico (LM1) the agglutination was very strong.

Only the isolates from Peru agglutinated with the *V. villosa* lectin at the maximum concentration assayed. With the WGA lectin at a concentration of 5 μg/ml, only the reference strain *L.* (*V.*) *peruviana* agglutinated. The three isolates from Spain and the LP4 isolate also agglutinated with WGA, but agglutinated, despite that the concentrations were close to the maximum assayed. *A. hypogaea* lectin agglutinated with all the isolates, but with differences in the concentration required for agglutination.

Fig. 2 shows the isoenzymatic profiles of the isolates analyzed by six enzyme systems (GPI, IDH, MDH, ME, SOD, and PGM). The isoenzymatic pattern of some of the isolates studied appears to reveal variations in the number of bands and their isoelectric points. This was the case of *L.* (*L.*) *donovani*, for which the profile with the six systems studied, in general, differed from the rest. The isolate LP4 was also different from the rest of the strains.

The pattern of *L.* (*V.*) *peruviana* (Fig. 2, lane 1), differed from the other isolates from Peru (Fig. 2, lanes 3, 7–9); nevertheless, the isolates LP2 and LP3 showed profiles very similar to the enzymes IDH, ME, and SOD (Figs. 2B, D, and E, lanes 7 and 9, respectively).

The isolates from Mexico had isoenzymatic profiles similar to each other, although the variations between LM2 and the other two isolates in the number of bands was apparent in the enzyme GPI (Fig. 2A). The isolates LM1 and LM3 were similar in the enzymes IDH, ME, SOD, and PGM (Figs. 2B, D–F). The isolate LM2 was similar to LM1 and LM3 only in SOD. Variations are observed among all isolates in GPI and MDH (Figs. 2A and C).

The DNA of the kinetoplast of the eight isolates and the four reference strains were purified and then digested with four restriction enzymes (*Hae*III, *Hin*fI, *Bam*HI, and *Eco*RI). Electrophoresis using agarose gel revealed that while intact kDNA was not able to penetrate the gel (Fig. 3E, lane 17), the smaller DNA restriction fragments that resulted after digestion did penetrate the gel, and a complete cleavage of kDNA by the endonucleases was characterized by a total disappearance of DNA from the top of the gel (Fig. 3). Only *Hin*f proved incapable of digesting all the kDNA of the four reference strains and the eight new isolates (Fig. 3B).

The restriction cleavage patterns of the reference strains *L.* (*V.*) *peruviana* and *L.* (*L.*) *donovani* were completely different from each other and from the strains *L.* (*L.*) *infantum* I and II as well as from the eight new isolates, with the 4 endonucleases (Figs. 3A–D, lanes 1 and 2). The *L.* (*L.*) *infantum* strains and the LS1 isolate presented homogeneous restriction profiles, although different from the other isolates (Figs. 3A–D; lanes 4–6); the same was true of the three Mexican isolates (Fig. 3, lanes 10–12), although the enzyme *Bam*HI was not able to digest the kDNA of these isolates.

The Peruvian isolates LP2, LP3, and LP4 presented very heterogeneous profiles. LP3 and LP1 were found to be homogeneous in sequence for the restriction enzymes tested (Fig. 3, lanes 7 and 8). The LP2 isolate somewhat resembled the LP3 and LP1 isolates, with the enzymes (*Hae*III and *Bam*HI) (Figs. 3A and C, lane 9), but more closely resembled the profile presented by the LP4 isolate, when its kDNA was digested by the enzyme *Eco*RI (Fig. 3D, lane 9).

The metabolites excreted by the parasite during its in vitro growth are shown in Fig. 4. All the isolates excreted pyruvate and acetate as main metabolites, although quantitative differences were evident between the isolates. For example, for LP4 and LM1 (Figs. 4B and G) the main metabolite was acetate, followed by pyruvate, while for the rest of the isolates the main metabolite was pyruvate followed by acetate (Figs. 4C–F). Some isolates,

Table 1
Agglutination activity of lectins for eight *Leishmania* isolates

Leishmania	Minimal concentration required to agglutinate (μg/ml)[a]			
	Concavalina ensiformis (Con A)	*Vicia villosa* (VV)	*T. vulgaris* (WGA)	*Arachis hypogaea* (PNA)
L. (*V.*) *peruviana*	1 (+)	1000 (+)	5 (+)	1 (+)
L. (*L.*) *donovani*	20 (++)	—	—	10 (+++)
L. (*L.*) *infantum* I	5 (+)	—	500 (+)	1000 (+)
L. (*L.*) *infantum* II	5 (+)	—	1000 (+)	1000 (+)
LS1	5 (+)	—	1000 (+)	1000 (+)
LP1	20 (+++)	1000 (+)	—	50 (+)
LP2	20 (+++)	1000 (+)	—	10 (+++)
LP3	20 (+++)	1000 (+)	—	1 (+)
LP4	20 (+)	—	1000 (+)	10 (+)
LM1	20 (+++)	—	—	1000 (+)
LM2	20 (+)	—	—	1000 (+)
LM3	20 (+)	—	—	1000 (+)

[a] Agglutination was scored from – (no agglutination at 1000 μg/ml) to (+++) (virtually complete agglutination). Average of three experiments.

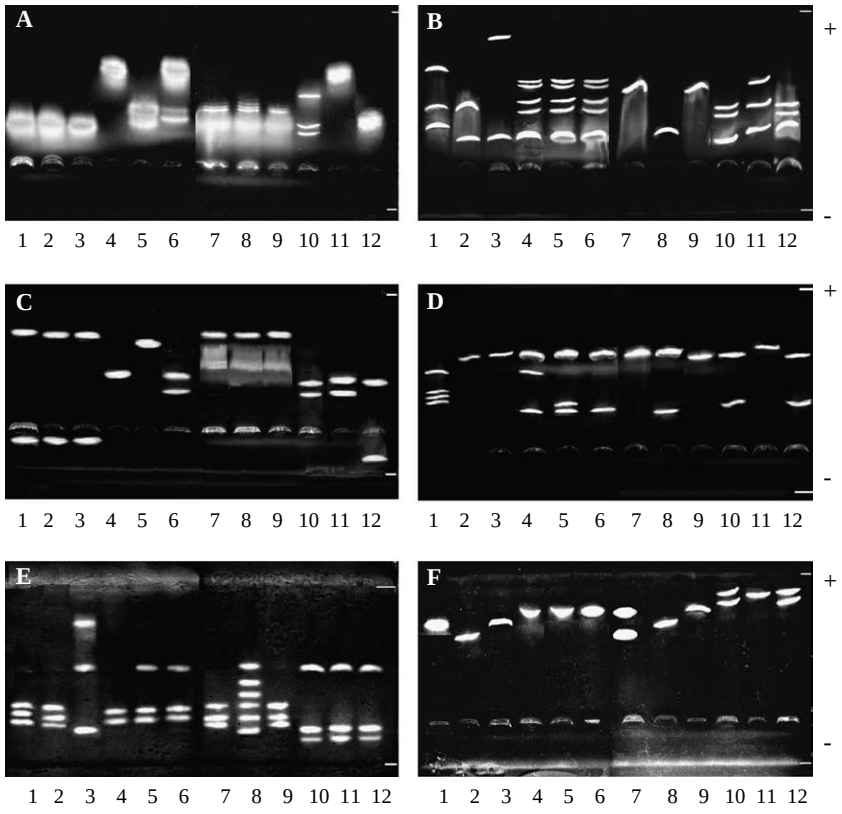

Fig. 2. Isoenzyme profiles of *Leishmania* isolates. Organisms: (lane1) *L.* (*V.*) *peruviana*; (lane 2) *L.* (*L.*) *donovani*; (lane 3) isolate LP4; (lane 4) *L.* (*L.*) *infantum* I; (lane 5) *L.* (*L.*) *infantum* II; (lane 6) isolate LS1; (lane 7) isolate LP3; (lane 8) isolate LP1; (lane 9) isolate LP2; (lane 10) isolate LM1; (lane 11) isolate LM2; and (lane 12) isolate LM3. Enzymes: (A) Glucose phosphate isomerase (GPI). (B) Isocitrate dehydrogenase (IDH). (C) Malate dehydrogenase (MDH). (D) Malic enzyme (ME). (E) Superoxide dismutase (SOD), and (F) Phosphoglucomutase (PGM).

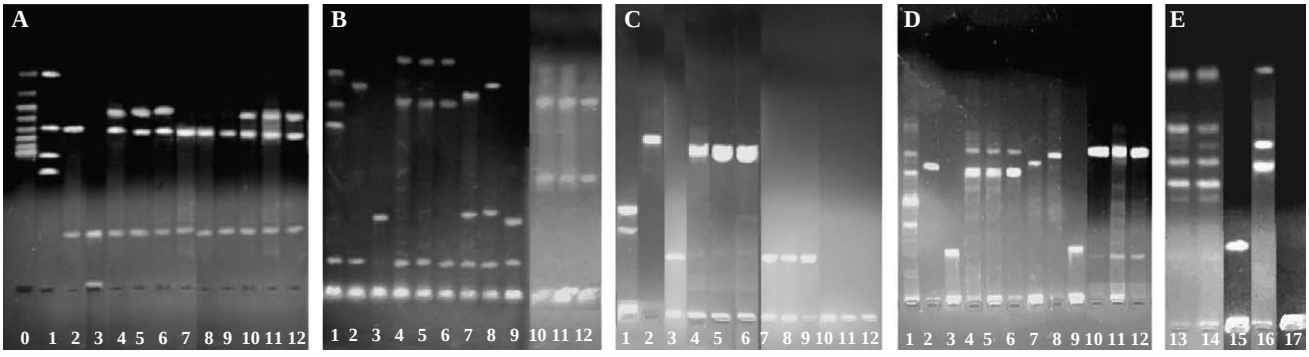

Fig. 3. Restriction endonuclease analysis of kDNA of *Leishmania* isolates. (A) kDNAs + *Hae*III. (B) kDNAs + *Hin*fI. (C) kDNA + *Bam*HI. (D) kDNA + *Eco*RI. Lane 1, *L.* (*V.*) *peruviana*; lane 2, *L.* (*L.*) *donovani*; lane 3, isolate LP4; lane 4, *L.* (*L.*) *infantum* I; lane 5, *L.* (*L.*) *infantum* II; lane 6, isolate LS1; lane 7, isolate LP3; lane 8, isolate LP1; lane 9, isolate LP2; lane 10, isolate LM1; lane 11, isolate LM2; and lane 12, isolate LM3. (E) Controls: kDNA of *Trypanosoma cruzi* strains *maracay*. Lane 13, kDNAs + *Hae*III; lane 14, kDNAs + *Hin*fI; lane 15, kDNA + *Bam*HI; lane 16, kDNA + *Eco*RI; and lane 17, Intact kDNA *L.* (*L.*) *donovani*. Size markers are 100-bp DNA ladder fragments (lane 0).

apart from excreting L-alanine as a secondary metabolite (Figs. 4B–C and F–G); (*L.* (*L.*) *donovani*, LP1, and LM1), also excreted a certain quantity of succinate. The three isolates from Spain showed some very similar spectra (data not shown), and a similar situation occurred with the isolates from Peru, as *L.* (*V.*) *peruviana* resembled the isolate LP4, and the rest of the Peruvian isolates were very similar to each other, differing only in the heights of the peaks (data not shown).

4. Discussion

To prevent variability derived from the culture medium, all the isolates were cultured in the same type of medium. The isolates were cultured in different cultured media having been assayed to identify the most adequate one. The MEM gave the highest growth density for most of the isolates and was selected to grow the isolates.

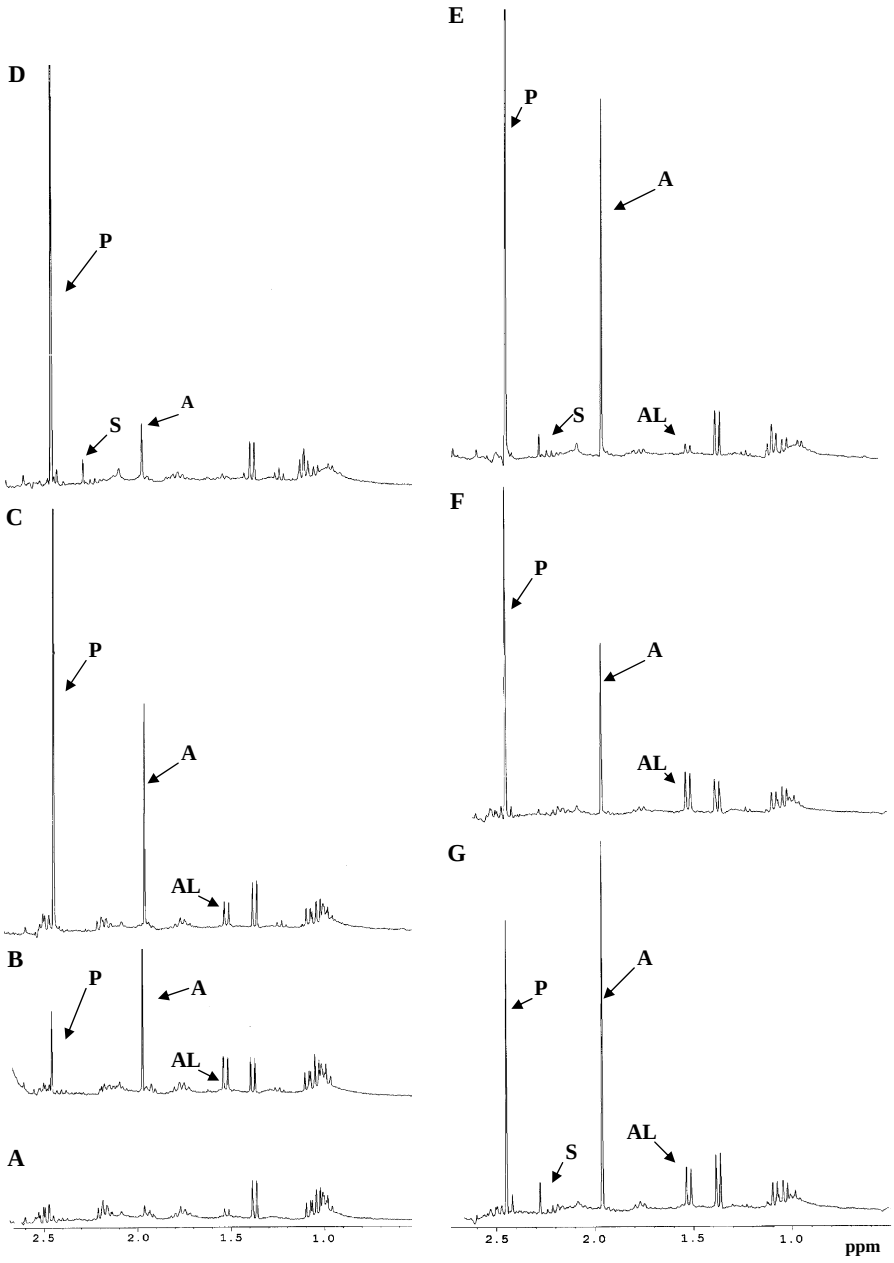

Fig. 4. ^{1}H NMR spectra of the *Leishmania* culture medium. (A) Fresh culture medium before inoculation of cells, (B) isolate LP4, (C) *L.* (*L.*) *infantum* I, (D) *L.* (*L.*) *donovani*, (E) isolate LP1, (F) isolate LM2, and (G) isolate LM1. **P**, pyruvate; **A**, acetate; **AL**, L-alanine; and **S**, succinate.

After determining the most suitable medium, we characterized all the isolates which identified to the genus *Leishmania*, by agglutination tests with lectins, by isoenzymatic analysis, by kDNA analysis and by the metabolic strategies of *Leishmania* in degrading energy substrates.

Cell-surface carbohydrates of *Leishmania* play a key role in parasite entry and survival inside the macrophage and in the digestive tract of the sandfly vector. Lectins are useful tools to study the topography, fate, distribution, and function of glycoconjugates on and inside parasites due to their specific and well-characterized sugar-binding properties (Andrade and Saraiva, 1999).

Cell-surface carbohydrates of different *Leishmania* species have been studied by a number of authors, taking advantage of their lectin-binding characteristics (Jacobson, 1994; Jacobson and Doyle, 1996). In the present study, all the isolates were agglutinated by Con A, at different concentrations, and results are in agreement with Dwyer (1977). The binding of WGA by *L.* (*L.*) *donovani* reported by Bandyopadhyay et al. (1991), was not observed in this study and other studies (Andrade and Saraiva, 1999; Sacks et al., 1995), although agglutination was observed with the Spanish *Leishmania* isolates as well as the LP4 and *L.* (*L.*) *peruviana* isolates. Our data coincide with those reported by different other authors (Andrade and Saraiva, 1999; Shottelius, 1982) in that all the isolates agglutinate with the PNA lectin.

According to the statistical analysis of Group Average Method, the Euclidean distance of the lectin-agglutination

test grouped the different isolates into four clusters: first *L.* (*L.*) *donovani*, the isolates from Mexico (LM1, LM2, and LM3) together with the Peruvian isolate LP4; second, the isolate from Spain (LS1), and the two reference strains of *L.* (*L.*) *infantum;* third, the isolates from Peru (LP1, LP2, and LP3); and fourth, the reference strain *L.* (*V.*) *peruviana* (data not shown).

Also, the isolates were characterized with six enzymes that can accurately identify parasites in the genus *Leishmania* (Shamsuzzaman et al., 2000). When the statistical study was made on the basis of the results from the isoenzyme-pattern comparisons, the different isolates were grouped again in four clusters: cluster I with *L.* (*V.*) *peruviana*; cluster II with *L.* (*L.*) *donovani* and the Peruvian isolates (LP2, LP3, and LP4); cluster III with the Spanish strain *L.* (*L.*) *infantum* I, *L.* (*L.*) *infantum* II and the isolate LS1 together with the Mexican isolates and cluster IV with Peruvian isolate LP1.

When the statistical study was made on the basis of the restriction cleavage patterns, the isolates were grouped into four clusters. Cluster 1 contained *L.* (*V.*) *peruviana*, cluster 2 the LP4 isolate, and cluster 3 *L.* (*L.*) *donovani*; cluster 4 could be divided into three subclusters, the first included the two *L.* (*L.*) *infantum* strains and the LS1 isolate, the second included the LP1, LP2, and LP3 isolates, and the third included the three Mexican isolates.

It is known that members of the family Trypanosomatidae are incapable of completely degrading carbohydrates even in the presence of oxygen, producing CO_2 and dicarboxylic acids. The relative proportion of these end-products varies among subspecies (Miralles et al., 2002; Urbina et al., 1993).

The ^{1}H NMR study indicated that the main metabolites by all the isolates excreted were pyruvate and acetate. Some isolates the secondary metabolites were succinate and in others alanine. The Group Average Methods statistical treatment grouped the isolates into three clusters: first, *L.* (*V.*) *peruviana* and LP4; second, *L.* (*L.*) *donovani*; and third, the rest of the isolates (data not shown).

The most appropriate identification and phylogenetic relationship was achieved with the analysis of the all data pooled, providing the following grouping according to the statistical method used (Fig. 5): the cluster analysis gave two clear groups that in turn could be broken down into subgroups. The first large group (cluster 1) was composed of three subgroups: the first would be *L.* (*V.*) *peruviana* alone; the second would be the Spanish strains, two of which had already been classified as belonging to *L.* (*L.*) *infantum*, and our data indicate that the third strain (LS1) also belongs to one species; and the third subgroup would be from Mexico, which according to our data would belong to the same strain. The second large cluster is formed by the Peruvian strains (LP1, LP2, and LP3), this not being surprising as these strains were isolated in the same geographical area and present similar chronic cutaneous lesions, compromising the mucosa ones, and the strain *L.* (*L.*) *donovani* is phylogenetically closer to the Peruvian strains.

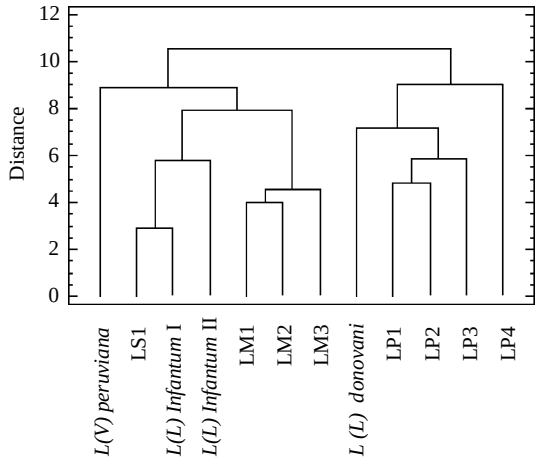

Fig. 5. Dendrogram based on individual hierarchical cluster analysis (Program Stat-Graphics version 5.0).

Finally, farther removed and forming a different subgroup is the LP4 isolate of the department of Cajamarca, which would belong to a different strain from *L.* (*V.*) *peruviana* and from the other three Peruvian isolates, and presumably for their geographic location would be closer to an Ecuadorian or Colombian strain.

All these groupings establish a certain correlation between the geographical distribution of the isolates with the identity of the species. Nevertheless, it is necessary to probe further into this matter in order to establish whether the Peruvian isolates present three different species: one would be *L.* (*V.*) *peruviana*, another the strain isolated in Cajamarca (LP4) (department bordering Ecuador and Colombia); and the third would include the three strains isolated from the department of La Libertad, which would be considered a different strain or subspecies, also with different clinical manifestations. In addition, it is necessary to include the reference strain *L.* (*L.*) *mexicana* to confirm that the three isolates from Mexico belong to it or are very closely related to it.

Acknowledgment

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Identification of New World *Leishmania* species from Peru by biochemical techniques and multiplex PCR assay

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Leishmania species; Peru; isoenzymatic characterization; kDNA analysis by restriction enzymes; multiplex PCR assay.

Abstract

We have characterized diverse strains or species of *Leishmania* isolated in humans that are currently circulating throughout Peru, by means of isoenzymatic characterization, kDNA analysis by restriction enzymes, and multiplex PCR assay. The cluster analysis gave five groups. Cluster 1 includes *L.* (*L.*) *donovani* together with the isolates LP4 and LP7, forming the *donovani* complex. Thus, this complex corresponds to the New World visceral form, *L.* (*L.*) *chagasi*. Cluster 2 is formed by the isolates LP1–LP3, LP6, LP10, LP9, and LP11, phylogenetically intermediate between Cluster 1 and Cluster 3, or they can be treated as hybrids. Cluster 3 is divided into two subgroups: one formed by *L.* (*V.*) *peruviana*, together with the isolates LP14 and LP5, and the second one formed by *L.* (*V.*) *brazilensis* and the isolate LP8. These two subgroups form part of the *brazilensis* complex. The three strains of *L.* (*L.*) *infantum* [*L.* (*L.*) *infantum* I and II and la LSI] make up Cluster 4. In Cluster 5, we include the three Mexican strains (LM1–LM3) forming one subgroup while we would place *L.* (*L.*) *amazonensis* in another subgroup. These two subgroups would comprise the complex *mexicana*.

Introduction

Parasitic protozoa of the genus *Leishmania* cause a broad spectrum of disease in humans throughout tropical and subtropical regions worldwide and are considered a major public health problem (Harris *et al*., 1998). Their clinical spectrum goes from cutaneous ulcers that heal spontaneously to serious visceral infections that can compromise the life of the patient, and between these two poles lies a wide range of clinical possibilities (Gallego, 2004). Leishmaniasis represents a serious obstacle for the socio-economic development of 88 countries, this being recognized by the World Health Organization as a worldwide health problem (World Health Organization, 1990). The 72 developing countries affected by this disease include Peru, where leishmaniasis constitutes the third cause of morbidity from transmitted diseases, after malaria and tuberculosis (Ministerio de Salud del Perú, Dirección General de Salud de las Personas, Dirección del Programa de Control de Enfermedades Transmisibles, 1995). It is a parasitosis in continuous expansion: 4645 cases were reported in 1999, and 5998 in 2002 (Cáceres & Montoya, 2002). In Peru, leishmaniasis is endemic in the Andes valleys as well as on the Amazonic plain. They present two main forms of tegument leishmaniasis, defined primarily by geographical and clinical characteristics: Andean leishmaniasis or Uta, and jungle leishmaniasis or Espundia (Lucas *et al*., 1998).

In the Americas, two taxonomic groups of *Leishmania* exist, the subgenera *Leishmania leishmania* and *Leishmania viannia*; the subgenus *viannia*, which is also known as the *brazilensis* complex, includes the species *Leishmania brazilensis*, *Leishmania peruviana, Leishmania panamensis*, and *Leishmania guyanensis*, while the subgenus *Leishmania* may be further divided into species complexes: the *mexicana* complex (*Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania garnhami*, *Leishmania aristidesi,* and *Leishmania pifanoi*), and the *donovani* complex (*L. chagasi*, Laison & Saw, 1987).

In Peru, five *Leishmania* species have been identified: *L. (Leishmania) amazonensis, L. (Viannia) guyanensis,* and *L. (V.) braziliensis* are etiological agents of cutaneous and

mucocutaneous leishmaniasis (Espundia) in the Amazon region; *L. (V.) peruviana* causes Andean cutaneous leishmaniasis (Uta) (Rodriguez, 2000); and, in provinces situated to the east of the Andes, *Leishmania (V.) lainsoni* is the pathogen (Lucas *et al.*, 1994).

Leishmania species are morphologically very similar and species identification is possible using standard biochemical methods (lectin agglutination, isoenzyme analysis, analysis of kinetoplast DNA (kDNA) restriction fragment using different restriction endonuclease, etc.) (Andrade & Saraiva, 1999; Shamsuzzaman *et al.*, 2000; Belhadj *et al.*, 2003; Sampali *et al.*, 2003; Rodríguez-González *et al.*, 2006). The ability to distinguish between *Leishmania* species is crucial when prescribing treatment as well as when determining possible control measures in epidemiological studies. Frequently, *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. However, geographical origin is an inadequate criterion in nonendemic areas, as well as endemic regions where multiple species of *Leishmania* may coexist. Identification of the infecting species based on clinical symptoms can be problematic, because several species cause both cutaneous and mucocutaneous disease while others cause visceral and cutaneous disease (Schönian *et al.*, 2003).

In a recent publication, we characterized eight *Leishmania* promastigotes isolated from different geographical areas: Peru, Mexico, and Spain (Rodríguez-González *et al.*, 2006). The stocks were characterized by carbohydrate cell-surface residues using agglutinations with lectins, by isoenzyme analysis, and by analysis of kDNA restriction fragment length polymorphism using four different restriction endonucleases. These isolates were compared with four reference stocks and typified as *Leishmania* (*L.*) *donovani*, two stocks of *Leishmania* (*L.*) *infantum*, and one species of *L.* (*V.*) *peruviana*. The Spanish isolate was identified as belonging to *L. (L.) infantum*, the stocks from Mexico as the Mexican complex, and the four isolates from Peru as *L. (V.) peruviana* (Rodríguez-González *et al.*, 2006). On this occasion, we characterized 10 new stocks, all isolated in humans from different regions of Peru, by the National Health Institute of Lima. The characterization was made by isoenzyme analysis, restriction kDNA analysis using different restriction endonucleases, and single-step multiplex PCR assay. In the same way, we have included for the PCR assay those isolates characterized in the previous work (Rodríguez-González *et al.*, 2006).

Materials and methods

Parasite isolation and *in vitro* culture

The 10 stocks were isolated from different areas of Peru. The stock that we call LP5 was isolated from a male 20 years of age who presented a cutaneous jungle lesion in the year 2001. Stocks LP6 and LP7 were also isolated from males having cutaneous lesions, LP8 being from a male 47 years of age with a cutaneous lesion on the back in the year 2002. These four isolates came from the Peruvian jungle region. Stocks LP9 and LP10 came from males of the Andes area of Peru (Department of Huanuco) bearing cutaneous lesions and were isolated in the year 2002. Stock LP11 came from the same region, but was isolated from a male 73 years of age with a cheek lesion (verrucosa form) and an ulcerous lesion on the leg of 5 months development. Stocks LP12, LP13 and LP14 were from males living on the border between Colombia and Ecuador, all presenting cutaneous lesions on the right and left temple. All these isolates were sent to our laboratory from the Lima National Health Institute (Peru). The stocks (from LP5 to LP14) isolated were cloned and cultured *in vitro* in MTL medium plus 10% inactivated foetal bovine serum kept in an air atmosphere at 28 °C (Sánchez-Moreno *et al.*, 1995).

Stocks LP1–LP4, LM1–LM3 and LS1, their isolation, and origin are described in Rodríguez-González *et al.* (2006). For reference strains, we used *L. (L.) donovani* (LCR-L 133, Leishmania Reference Center Jerusalem, Israel) isolated in a human case of kala-azar in Begemder (Ethiopia), *L. (V.) peruviana* (MHOM/PE/1984/LC26), two strains of *L. (L.) infantum* I and II, characterized as MCAN/ES/2001/UCM-10 and MCAN/2000/UCM-1, respectively isolated in Spain, *L.* (*L.*) *amazonensis* (MHOM/BR/1973/M1845) and *L.* (*V.*) *brazilensis* (MHOM/BR/1975/M2904). The reference strains have been maintained for several years in our laboratory by successive passes in cultures of NNN medium modified with a liquid phase of minimal essential medium (MEM) plus 10% inactivated foetal bovine serum kept in an air atmosphere at 28 °C. To maintain infectivity, a subculture was made approximately every 2 weeks and at was inoculated in the Syrian golden hamster, *Mesocricetus auratus*, least every 6 months, and parasites were isolated from the spleen 30–45 days postinoculation. The isolates from hamsters were cloned and cultured *in vitro* as previously described (Sánchez-Moreno *et al.*, 1995).

Isoenzyme characterization

Crude homogenates were obtained from 300 mL of culture medium containing 2×10^7 cells mL^{-1}. Cells were harvested by centrifugation at $1500\,\boldsymbol{g} \times 10$ min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme stabilizer solution containing 2 mM dithiothreitol, 2 mM E-aminocaproic acid, and 2 mM EDTA (Fernández-Ramos *et al.*, 1999). The samples were frozen at −80 °C for 15 min and thawed at 25 °C. After several freezing–thawing cycles, cell lysates were centrifuged at $8000\,\boldsymbol{g} \times 20$ min at 4 °C, and the supernatants were stored

in liquid nitrogen until use. The protein concentration was determined using the Bradford method and stored at a final concentration of 1 mg mL^{-1} of protein.

The enzymes were separated by isoelectric focusing in a PhastSystem apparatus, using Phast-gel IEF 3-9 (Pharmacia, Freiburg, Germany).

The following enzymes were tested: malic enzyme (EM), glucose 6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), and superoxide dismutase (SOD). The staining procedures are described in Fernández-Ramos *et al.* (1999).

kDNA isolation

Promastigotes were collected by centrifugation of 300 mL of culture medium, when their concentrations had reached about 2×10^7 cells mL^{-1}, after about 5 days. They were washed twice in 50 mL of 0.15 M NaCl, 0.015 M sodium citrate, and once with SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). kDNA was obtained according to the procedure described by Gonçalves *et al.* (1984).

Restriction-enzyme digestion and electrophoresis analysis

The kDNA extracts (3 μg mL^{-1}) were completely digested with restriction endonucleases (HaeIII, BamHI, HinfI and EcoRI, and MspI) according to the manufacturer's prescribed buffer conditions (Boehringer-Ingelheim, Barcelona, Spain). The digestion products were electrophoresed in 1.3% agarose slab gels as described elsewhere (Riou & Yot, 1977) and the fragment sizes were estimated by comparing their mobilities with those of a 100-bp DNA ladder (Gibco-BRL, Gaithersburg). The gels were stained with ethidium bromide (10 μg mL^{-1} for 10 min) and photographed under UV light with a Polaroid camera (665 film).

Primer design and PCR amplification

Sequences for the SL RNA region of the New World *Leishmania* species were aligned with GENEJOCKEY II software (Bosoft, Ferguson, MO) to identify potential sites for genus-specific as well as complex-specific PCR priming (Harris *et al.*, 1998). We chose oligonucleotide primers that were either conserved in all *Leishmania* species (LU-5A) or specific to each New World complex (LB-3C, LM-3A, and LC-3L). The primers and their sequences are as follows: LU-5A, 5′-TTTATTGGTATGCGAAATTC-3′; LB-3C, 5′-CGT(C/G)CCGAACCCCGTGTC-3′; LM-3A, 5′-GCACCG CAC CGG(A/G)CCAC-3′; and LC-3L, 5′-GCCCGCG (C/T)GTCACCACCAT-3′. Oligonucleotides were synthesized by Thermo Electron Corporation (Germany).

We prepared a 50-μL reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each deoxynucleotide triphosphate, 15 mM $MgCl_2$, 10.5% dimethyl sulphoxide, 50 mM tetramethylammonium chloride, 0.4 μM 5′ primer LU-5A, 0.2 μM of each 3′ primer (LB-3C, LM-3L, and LC-3L), 0.04 U of Taq DNA polymerase (Promega), and 10 ng of kDNA preparation. An initial denaturing step of 95 °C for 5 min was followed by 35 cycles of 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR products were electrophoresed in a 1.3% agarose gel and stained with ethidium bromide.

Statistical study

The statistical methods were based on individual hierarchical cluster analysis, selecting the Euclidean distance to the square as the basis for measuring the associations between individuals. The Euclidean distance was calculated by the following grouping procedures of simple linkage (R_k 0.7355), average linkage among groups (R_k 0.7518), average linkage (R_k 0.7726), centroid method (R_k 0.7586), median method (R_k 0.7204), and the Ward method (R_k 0.7570). The cophenetic coefficient (R_k) measures the degree of distortion between relationships, means in terms of original distances between individuals and those existing at the end of the analysis. The one with the highest cophenetic correlation was chosen as the optimal method.

In the selection procedures, the average linkage between groups was considered using the coefficient R_k of Rand, which is an index of the similarity between classifications. This analysis was made with the STATGRAPHICS program, version 5.0.

Results

Leishmania isolates, cultured *in vitro* in MTL medium plus 10% inactivated foetal bovine serum, reached cell densities on the order of 2×10^7 cells mL^{-1}. Figure 1 shows isoenzymatic profiles of the isolates analysed by seven enzyme systems (EM, G6PDH, IDH, MDH, GPI, PGM, and SOD). The isoenzymatic pattern of some of the isolates studied appears to reveal variations in the number of bands and their isoelectric points. This was the case of *L.* (*V.*) *braziliensis*, for which the profile with the seven systems studied, in general, differed from the rest of the stocks (Fig. 1c–e and g, lane 1); *L.* (*L.*) *amazonensis* was differentiated from the rest of the new isolates with the enzymes IDH and MDH (Fig. 1c and d, lane 2). The new isolate LP11 presented a similar profile for the enzyme MDH with *L.* (*L.*) *amazonensis* (Fig. 1d, lane 9). This same new isolate presented great differences with respect to the other isolates with the enzymes IDH and SOD (Fig. 1c and g, lane 9). Stocks LP6, LP13, and LP14 shared the same profile as *L.* (*L.*) *amazonensis* for malic

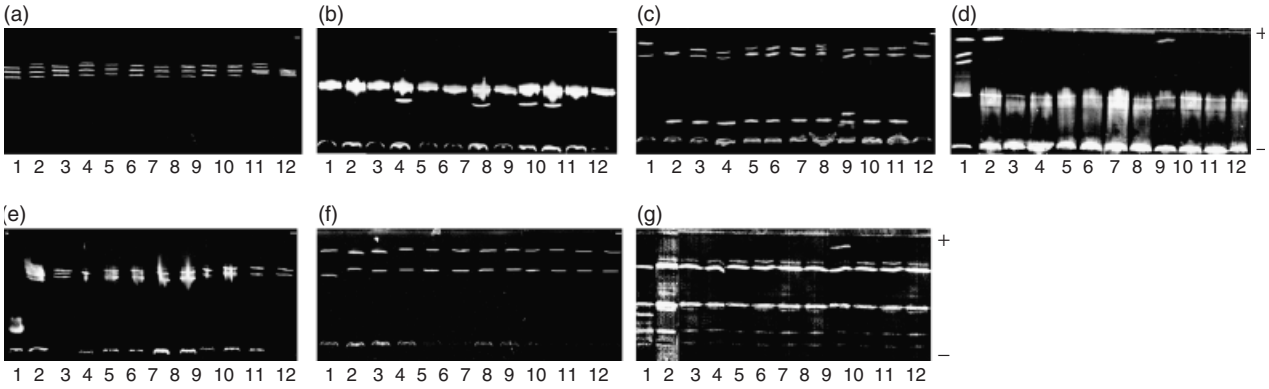

Fig. 1. Isoenzyme profiles of *Leishmania* isolates. Organisms: (1) *L. (V.) brazilensis*; (2) *L. (V.) amazonensis*; (3) isolate LP5; (4) isolate LP6; (5) isolate LP7; (6) isolate LP8; (7) isolate LP9; (8) isolate LP10; (9) isolate LP11; (10) isolate LP12; (11) isolate LP13; and (12) isolate LP14. Enzymes: (a) malic enzyme (ME); (b) glucose 6-phosphate dehydrogenase (G6PDH); (c) isocitrate dehydrogenase (IDH); (d) malate dehydrogenase (MDH); (e) glucose phosphate isomerase (GPI); (f) phospho-glucomutase (PGM); and (g) superoxide dismutase (SOD).

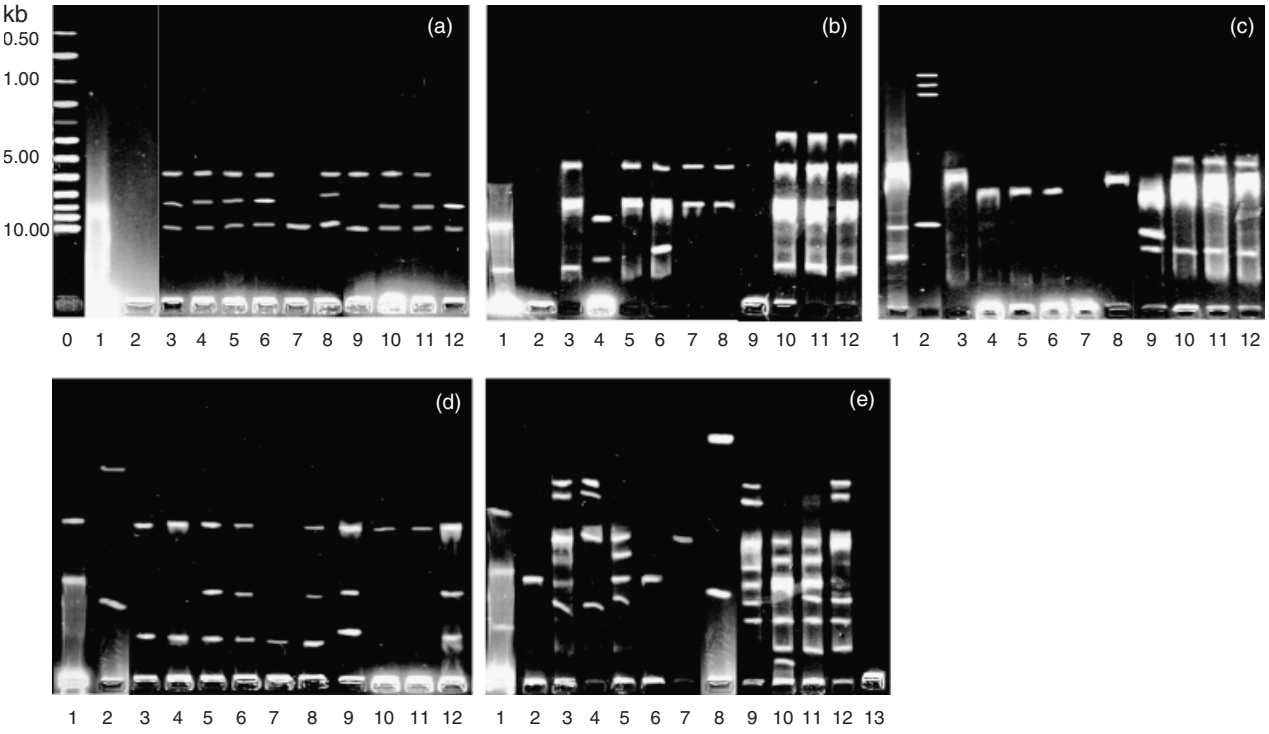

Fig. 2. Restriction endonuclease analysis of kDNA of *Leishmania* isolates. (a) kDNA+BamHI; (b) kDNAs+HinfI; (c) kDNAs+HaeIII; (d) kDNA+EcoRI; and (e) kDNA+*Msp*I. Lane (1) *L. (V.) brazilensis*; (2) *L. (V.) amazonensis*; (3) isolate LP5; (4) isolate LP6; (5) isolate LP7; (6) isolate LP8; (7) isolate LP9; (8) isolate LP10; (9) isolate LP11; (10) isolate LP12; (11) isolate LP12; (12) isolate LP14; and Lane 13: Intact kDNA *L. (V.) brazilensis*. Size markers are 1 Kb DNA ladder fragments (Lane 0).

enzyme (Fig. 1a, lanes 2, 4, 11, and 12). Stocks LP6, LP9, LP11, and LP12 with glucose 6-phosphate dehydrogenase enzyme were clearly differentiated from the two reference strains and the rest of the isolates (Fig. 1b, lanes 4, 7, 9, and 10). The enzyme PGM did not enable the detection of any significant difference between any of the isolates (Fig. 1f).

The kDNA of the 10 isolates and the two reference strains were purified and then digested with five restriction enzymes (BamHI, HinfI, HaeIII, EcoRI, and MspI). Electrophoresis using agarose gel revealed that while intact kDNA was not able to penetrate the gel (Fig. 2e, lane 13), the smaller kDNA restriction fragments that resulted after

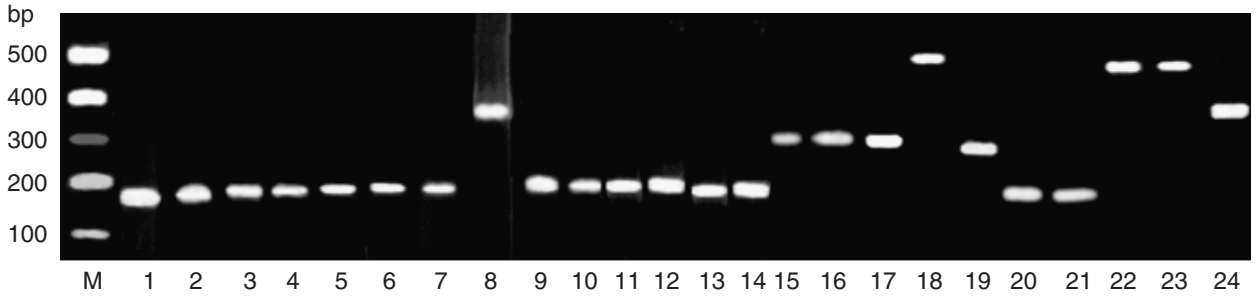

Fig. 3. Amplification products of the PCR multiplex assay from different *Leishmania* species and isolates using the primers LU-5A, LB-3C, LM-3A, and LC-3L. In each lane 10 μL of sample was placed. Lanes 1–14, LP1–LP14, respectively. Lane 15, isolate LM3; lane 16, isolate LM2; lane 17, isolate LM1; lane 18, isolate LS1; lane 19, *L. (V.) amazonensis*; lane 20, *L. (V.) peruviana*; lane 21, *L. (V.) brazilensis*; lane 22, *L. (L.) infantum* I; lane 23, *L. (L.) infantum* II; lane 24, *L. (L.) donovani*. Lane M, 100-bp DNA ladder; the lower band shown is 100 bp.

digestion did penetrate the gel, and a complete cleavage of kDNA by the endonucleases was characterized by a total disappearance of DNA from the top of the gel (Fig. 2). Not all the enzymes were capable of completely digesting the kDNA of the different isolates; for example, the enzymes BamHI (Fig. 2a) and HinfI (Fig. 2b) were incapable of completely digesting the kDNA of *L.* (*V.*) *brazilensis*, *L.* (*L.*) *amazonensis,* and the kDNA of the isolates LP6 and LP11.

The restriction-cleavage patterns of the reference strains *L.* (*V.*) *brazilensis* and *L.* (*L.*) *amazonensis* were completely different from each other with the five restriction enzymes, and both differed from the rest of the stocks. The isolates LP5–LP10 presented very homogeneous profiles with the enzymes BamHI, HinfI, and EcoRI (Fig. 2a, b and d, lanes from 3 to 8, both inclusive). Meanwhile, stock LP11 showed marked differences in the fragments caused by the enzymes HinfI, HaeIII, and MspI (Fig. 2b, c and e, lane 9). The isolates LP12–LP14 presented homogeneous restriction profiles that differed from the other isolates (Fig. 2a–d, lanes 10–12).

Figure 3 presents the results corresponding to the amplification of the kDNA by multiplex PCR assay of the 10 new isolates, of the two reference stocks, and of the isolates characterized previously by isoenzyme analysis and by restriction enzymes (Rodríguez-González *et al*., 2006). An amplification band of 370–390 bp was visible in the case of the reference stock *L.* (*L.*) *donovani* (Fig. 3, lane 24) and for the isolate LP7 (Fig. 3, lane 8). The two reference stocks *L.* (*L.*) *infantum I* and *II* and the stock called LSI presented a band of 530–550 bp as a product of the PCR. The three stocks from Mexico (LM1–LM3) amplified a band of 250–270 bp common to the three isolates. The reference stocks *L.* (*V.*) *peruviana* and *L.*(*V.*) *brazilensis* and the stocks LP1–LP14, excepting the isolate LP7, had similar kDNA fingerprints, with a band of 160–170 bp (Fig. 3, lanes 20, 21, from 1 to 7 and from 9 to 14). The reference stock *L.*(*L.*) *amazonensis* gave a band of 250-bp as the PCR product (Fig. 3, lane 19).

Discussion

The prognosis of human leishmaniasis depends in part on the species of the infecting parasite. In field situations the isolation of *Leishmania* cultures for this species characterization is not always possible, especially as very few parasites are often present in lesions, blood, skin, organs, and sandflies. Moreover, isolation and culture protocols can select for particular clones from a heterogeneous population. It is highly desirable to develop sensitive molecular tools that are able to identify and type *Leishmania* directly (Breniere *et al*., 1999).

By isoenzymatic analysis, 10 *Leishmania* isolates were characterized from different areas of Peru: the isolates LP5–LP8 from the Peruvian jungle, stocks LP9–LP11 from the Andes area, and stocks LP12–LP14 from the border with Colombia and Ecuador. The characterizations were made with seven enzyme systems that can accurately identify parasites in the genus *Leishmania* (Shamsuzzaman *et al*., 2000). The statistical study was performed on the basis of the results from the isoenzyme-pattern comparisons, including in this study other previously characterized isolates from Peru: LP1–LP4 and the reference strain *L.* (*V.*) *peruviana* (Rodríguez-González *et al*., 2006). The different isolates were grouped again into three clusters: Cluster 1 with stocks LP1, LP2, LP4, and the reference stock *L.* (*V.*) *brazilensis*; Cluster 2 with *L.* (*V.*) *peruviana*, *L.* (*L.*) *amazonensis*; and the stocks LP5, LP7, LP8, LP13, and LP14; and Cluster 3 with the rest of the stocks LP3, LP6, and LP9–LP12.

When the statistical study was made on the basis of the restriction cleavage patterns, the isolates were regrouped into four clusters: Cluster 1 included the reference stock *L.* (*V.*) *peruviana* together with the three isolates from the border between Peru, Colombia, and Ecuador (LP12–LP14); Cluster 2 held *L.* (*L.*) *amazonensis*; Cluster 3 was composed of *L.* (*V.*) *brazilensis* and the isolates LP1–LP11, excepting LP9, which made up Cluster 4.

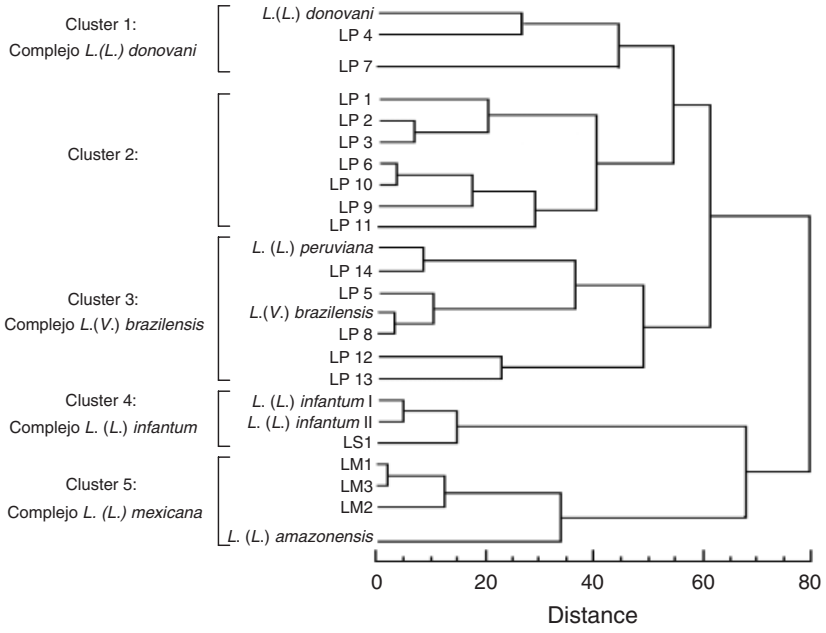

Fig. 4. Dendograma based on individual hirarchical cluster analysis (Program STAT-GRAPHICS version 5.0).

A multiplex PCR assay was developed and it uses a single conserved 5′ primer common to all *Leishmania* species plus three distinct 3′ primers, each of which is specific to a different complex (Harris *et al*., 1998). Thus, the identification of the *Leishmania* complexes was based on both the sizes of the products and the sequence specificities of the primers. For the *L.* (*L.*) *donovani*, products ranged in size from 370 to 390 bp, as in the case of the isolate LP7; for the three isolates from Mexico (LM1–LM3), the products ranged in size from 250 to 270 bp, and for *L.* (*V.*) *brazilensis* and the isolates LP1–LP6 and LP8–LP14, products ranged in size from 150 to 170 bp.

The PCR was expected to be specific only for the stocks belonging to the complexes *L.* (*L.*) *donovani*, *L.* (*V.*) *brazilensis*, and *L.* (*L.*) *mexicana*; however, with the PCR evaluation with stocks *L.* (*L.*) *infantum* I and II and the isolate called LS1, a common band was amplified to the three of 530–550 bp. This behaviour was presumably because the amplified regions were highly conserved in all the species, with some divergences, favouring the hybridization of the oligos (Cáceres & Montoya, 2002). The multiplex assay distinguished *Leishmania* from *Trypanosoma cruzi* and generated no product (data not shown). This is an important feature, because mixed infections of *Leishmania* and *T. cruzi* can occur and are subject to antibody cross-reactivities to related antigens in the two parasites (Harris *et al*., 1998).

When the statistical treatment is applied to these results, the isolates are grouped into three large Clusters. The first, in turn, is divided into two subclusters: (a) is *L.* (*L.*) *donovani* and isolate LP7, which may indicate that this isolate is *Leishmania* (*L.*) *chagasi*; (b) is comprised of *L.* (*L.*) *amazonensis* and the three Mexican stocks. The second large cluster was composed of two stocks of *L.* (*L.*) *infantum* I and II and the isolate LSI, which we can now identify as *L.* (*L.*) *infantum*. The third Cluster was made up of the reference stocks *L.* (*V.*) *brazilensis* and *L.* (*V.*) *peruviana* together with the rest of the isolates.

The most appropriate identification and phylogenetic relationship was achieved with the analysis of all the data pooled, including those from the previous study (Rodríguez-González *et al*., 2006). The following grouping of five clusters resulted according to the statistical method (Fig. 4). The first group (Cluster 1) contained *L.* (*L.*) *donovani* together with the isolates LP4 and LP7, which would comprise the *donovani* complex, these surely being species belonging to the New World visceral form (*L.* (*L.*) *chagasi*). Cluster 2 would be made up of LP1, LP2, LP3, LP6, LP10, LP9, and LP11; these would be phylogenetically intermediate between the *donovani* complex (Cluster 1) and the *brazilensis* complex (Cluster 3), or they could be hybrids, as is known to occur on the American continent (Hernández *et al*., 1991). This hybridization would not be surprising as these strains were isolated in the same geographical area and present similar chronic cutaneous lesions, compromising the mucosal ones, while the strain *L.* (*L.*) *donovani* is phylogenetically closer to the Peruvian strains (Rodríguez-González *et al*., 2006). Cluster 3 could be divided into two subgroups, one formed by *L.* (*V.*) *peruviana*, together with the isolates LP14 and LP5, and the second subgroup composed of *L.* (*V.*) *brazilensis* and the isolate LP8, these two subgroups forming part of the *brazilensis* complex (Laison & Saw, 1987). *L.* (*V.*) *peruviana* is known to be ecogenetically heterogeneous, and thus *L.* (*V.*) *peruviana* from the Department of Huancabamba (Piura) differs from

species originating in the south (Departments of Ancash, Lima, and Ica). There is a close relationship between the evolution of the species *L.* (*V.*) *peruviana* and *L.* (*V.*) *brazilensis*, although the latter is karyotypically more homogeneous than *L.* (*V.*) *peruviana*. Meanwhile, *L.* (*V.*) *brazilensis* and *L.* (*V.*) *peruviana* are genetically closer, as they can be distinguished only by the locus of one enzyme (Dujardin *et al.*, 1995).

Cluster 4 would be formed by the stocks *L.* (*L.*) *infantum* [*L.* (*L.*) *infantum* I and II and LSI]. Finally, Cluster 5 would include the three Mexican stocks (LM1–LM3) forming a subgroup, and another subgroup would have *L.* (*L.*) *amazonensis*, these two subgroups forming the *mexicana* complex. All these groupings establish a certain correlation between the geographical distribution of the isolates with the identity of the species.

New World *Leishmania* species of different complexes can be found in the same types of clinical specimen; for example, *L.* (*L.*) *mexicana* and *L.* (*L.*) *chagasi* are found in cutaneous nodules (Neva *et al.*, 1997), while *L.* (*V.*) *brazilensis* and *L.* (*L.*) *amazonensis* are found in lesions of cutaneous leishmaniasis (Grimaldi & Tesh, 1993), among others. Other studies suggest that the aetiology of the American visceral leishmaniasis in the New World may be more complex than currently believed. Most of the New World *Leishmania* species appear capable of producing a spectrum of disease manifestations (Hernández *et al.*, 1991). The different species of *Leishmania* are morphologically indistinguishable, and thus the taxonomy of this genus bears special medical importance. It is very difficult to establish the number of species existing in Peru for the continuous discovery of new species, including, in the last few years, hybrid forms (Mita, 2001; Flores *et al.*, 2002). Furthermore, near Brazil, Venezuela, Colombia, Chile, Bolivia, and Ecuador, a mixture of species has resulted from human migrations, urbanization, deforestation, etc., but much more from the animals that migrate through the Amazon, carrying parasites previously unknown in these areas (Davies *et al.*, 2000; Desjeux, 2004; Alcántara de Castroa *et al.*, 2005). Moreover, New World cutaneous and mucocutaneous leishmaniasis are increasingly diagnosed among European travellers returning from Latin American countries and the disease is usually acquired in rural or jungle areas (Schwartz *et al.*, 2006). Information on the incidence of cutaneous leishmaniasis in travellers is scarce for a number of reasons: (1) it is not a notifiable disease in most industrialized countries; (2) the number of exposed people (i.e. the total number of travellers to endemic areas) is often unknown; (3) the disease is frequently misdiagnosed; and (4) spontaneous self-healing is possible (Scope *et al.*, 2003). Latin America was the primary source of infection (Zeegelaaer *et al.*, 2005). The differentiation and characterization of these parasite populations are vital in order to establish better diagnosis, treatment, prognosis, and control of the parasitoses, as well as to understand the influence that intraspecific variations may have in the epidemiology of these diseases. In this work, we have characterized a series of human isolates of different stocks or species of *Leishmania* that are currently circulating throughout Peru using isoenzymatic characterization, by kDNA analysis by restriction enzymes, and PCR multiplex assay, which enables the phylogenetic study of these stocks and contributes to a rapid identification of *Leishmania* complexes for epidemiological purposes in areas where leishmaniasis is endemic. Today, PCR is the diagnostic method of choice since it has a high sensitivity and gives a species-specific diagnosis (Santamaría *et al.*, 2005; Schwartz *et al.*, 2006). Recently, several studies in Ecuador have found that PCR-based methods are more sensitive (85.4% on average) than classical diagnostic techniques, called slit smears (45.4%), culture (57.2%), and histopathology (34.7%) (Calvopina *et al.*, 2004).

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Identification of trypanosome strains isolated in Central and South America by restriction endonuclease cleavage and amplification using the polymerase chain reaction of kinetoplast-DNA

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Abstract

The exact identification of a given parasitic agent represents the key to a timely diagnosis of the disease in order to proceed with the correct treatment and control. Molecular biological techniques have arisen as useful tools for these aims. In this study, we report the characterization of seven trypanosome stocks isolated in different geographical areas of Latin America and from different vector species, by analysis of kinetoplast DNA (kDNA) restriction fragment-length polymorphism, using five different restriction endonucleases and a duplex PCR assay. Of the seven trypanosome stocks were analysed, one was from Mexico (TM5) and six from Peru, four of these isolated from different species of triatome (TP504, TP702, TP704 and TP706) and the other two isolated from the salivary glands of *Rhodnius ecuadorensis* (TRa605 and TRa606). Additionally, three strains of *Trypanosoma cruzi* isolates from a human case (strain TC-Maracay, TY and TCL) and one strain of *T. rangeli* (TRa, Cajamarca-Peru strain) characterized and maintained in our laboratory, were used as reference strains. According to the statistical study, the stocks were grouped into three clusters: cluster 1 held the three stocks of *T. cruzi* used as references. Very close to these were the stocks isolated in the Peruvian Amazon (TP702, TP704 and TP706), which constituted cluster 2. This cluster also included stock TP504, although this would be a hybrid between *T. cruzi* and *T. rangeli*. Cluster 3 consisted of the trypanosomes isolated from salivary glands (TRa, TRa605, TRa606 and TM5), these four stocks being the same as *T. rangeli*, and the phylogenetic separation observed could be due to having been isolated *T. cruzi.*

Keywords: Trypanosoma cruzi strains, characterization, kDNA analysis by restriction enzymes, multiplex PCR assay, Perú, México.

1. Introduction

Trypanosoma cruzi is the causal agent of Chagas disease, which is characterized by an acute phase after the initial infection and then a chronic phase in which there is a progressive degenerative disorder of the cardiac and/or gastrointestinal systems. Different isolates or *T. cruzi* stocks exhibit a great deal of heterogeneity in terms of isoenzyme patterns, surface antigens, nuclear-DNA content, morphological features, and kinetoplast minicircle DNA sequences [1].

During recent years, various research groups have demonstrated this broad biochemical, genetic, and molecular variability in populations of *T. cruzi* and *T. rangeli*, a species apparently not pathogenic for humans but very frequent in mixed infections by *T. cruzi* in some countries [2]. This heterogeneity and variability in its biological behaviour have hampered the establishment of suitable parameters for classification and taxonomy, making its characterization crucial in the Chagas disease clinic [3,4].

In a previous paper, we characterized seven trypanosomatids isolated from different geographical areas of South America [5], using standard methods for taxonomic purposes [6,7]: isoenzyme analysis and lectins with different specificities for surface-membrane carbohydrate residues. More recently, it has been reported that the use of kinetoplast minicircle DNA sequences, by means of analysing the restriction enzymes or by polymerase chain-reaction DNA from the kinetoplast for this type of study are more precise, reliable, and appropriate [8]. For this, in this new study, we have examined these seven isolates from Peru and Mexico by means of kDNA restriction fragment analysis and have amplified species-specific and possibly strain-specific fragments of minicircle kDNA. For comparison, we have used four isolates characterized as: *T. cruzi* stock Maracay, stock Y, stock CL, and *T. rangeli*.

2. Materials and methods

2.1. Parasites

The seven stocks isolated from different areas of South America: TM5 from *Rhodnius prolixus* in the Yucatan Peninsula of Mexico; the TP504 isolate of *Panstrongylus chinai* in the department of La Libertad in northern Peru; the TP702 and TP706, isolates of *Triatoma carrioni* and *T. herreri*, respectively, in the department of

Amazonas in north-eastern Peru; the TP704 isolate of *T. infestans* in the department of Arequipa in south-eastern Peru; and the TRa605 and TRa606 isolates, both from the salivary glands of two *Rhodnius ecuadoriensis* in the department of La Libertad, Peru. For comparison, we also included *T. cruzi* (TC) Maracay, originally isolated from a clinical case in the Malariological Division of Environmental Health of Maracay (Venezuela) strain; *T. cruzi* (TY) strain Y [9]; *T. cruzi* (TCL) strain CL [10], and *T. rangeli* (TRa, Cajamarca-Peru strain), isolated from the salivary glands of *Rhodnius ecuadoriensis* [7] and maintained in our laboratory by routine procedures [11].

After isolation, the new flagellates were transferred to the biphasic culture medium Nicolle Nocloe Novi and MacNeal medium, supplemented with minimal essential medium, and 20% inactivated foetal bovine serum. The isolates were cloned and cultured *in vitro* using Grace's medium (Sigma) [11]. The parasites were inoculated at a density of $1.6x10^6$ into 5 ml of medium in 25-ml flacon flasks, and cultured at 28ºC. Aliquots were taken every 24 h for 9 days and the number of parasites was counted in a Neubauer haemocytometer. On the last day, the cells were removed by centrifugation at 600 *g* for 10 min.

2.2. Kinetoplast-DNA isolation

Epimastigote forms were collected by centrifugation of 300 ml of culture medium when their concentrations had reached about 2 $x10^7$ cells /ml. They were washed twice in 50 ml of 0.15 M NaCl, 0.015 M sodium citrate and once with SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). Kinetoplast DNA was obtained according to the procedure described by Gonçalves et al. [12].

2.3. Restriction enzyme digestion and electrophoresis analysis

The kDNA extracts (1 µg/ml) were completely digested with restriction endonucleases (*Hae* III, *BamH* I, *Hinf* I, *Msp* I, and *EcoR* I) according to the manufacturer's prescribed buffer conditions (Boehringer-Ingelheim, Barcelona, Spain). The digestion products were electrophoresed in 1.5% agarose slab gels as described by [13], and the fragment sizes were estimated by comparing their mobilities with those of a 100-bp DNA ladder (Gibco-BRL, Gaithersburg, USA). The gels were stained with

ethidium bromide (10 µg/ml for 10 min) and photographed under UV light with a Polaroid camera (665 film).

2.4. Analysis of amplified minicircle kDNA

For polymerase chain reaction (PCR) assay, we used a MyCycler™ thermal cycler (Bio-Rad). The kDNA was obtained as described previously. For *T. cruzi* detection, we used a modified version of a duplex PCR assay targeted to the 189-bp telomeric junction (Tc189, Gen Bank accession number ***AF100651***). The primers were T189Fw2 (5'-CCAACGCTCCGGGAAAAC-3') and Tc189Rv3 (5'-GCGTCTTCTCAGTATGG ACTT-3'). For *T. rangeli* detection, we used an assay targeted to a conserved subtelomeric region (SubTr, GenBank accession number ***AF426020***). The primers were TrF3 (5'-CCCCATACAAAACACCCTT-3') and TrR8 (5'-TGGAATGACGGTGCG GCGAC-3') [14]. All oligonucleotides were purchased from Thermo Electron Corporation (Germany) and PCR amplifications were conducted in a final volume of 25 µl containing 0.2 mM deoxynucleoside triphosphate mixture, 1.5 mM $MgCl_2$, 50 mM KCI, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.24 mM deoxynucleoside triphosphate mix, 0.01% bovine serum a1bumin, 0.4 mM each Tc189Fw2 and Tc189Rv3 primers, 0.67 mM each TrF3 and TrR8 primers, 1.25 U of Taq DNA polymerase (Promega), and 10 ng of kDNA preparation. An initial denaturing step at 94ºC for 4 min was followed by 35 cycles of 30 s at 55ºC, 40 s at 72ºC, and 1 min at 94ºC. This amplification was followed by incubations of 1 min at 55ºC and 3 min at 72ºC. PCR products were electrophoresed in a 1.3% agarose gel, stained with ethidium bromide and photographed as above.

2.5. Statistical study

The statistical methods were based on individual hierarchical cluster analysis, selecting the Euclidean distance to the square as the basis for measuring the associations between individuals. The Euclidean distance was calculated by the following grouping procedures of: simple linkage (R_k 0.7355), average linkage among groups (R_k 0.7518), average linkage (R_k 0.7726), centroid method (R_k 0.7586), median method (R_k 0.7204), and the Ward method (R_k 0.7570). The cophenetic coefficient (R_k) measures the degree of distortion between relationships, means in terms of original distances between

individuals and those existing at the end of the analysis. The one with the highest cophenetic correlation was chosen as the optimal method.

In the selection procedures the average linkage between groups was considered, using the coefficient R_k of Rand, which is an index of the similarity between classifications. This analysis was made with the StatGraphics program, version 5.0.

3. Results

The DNA of the kinetoplast of the 7 isolates and the 4 reference strains were purified and then digested with five restriction enzymes (*EcoR* I, *BamH* I, *Hae* III, *Hinf* I, and *Msp* I). Electrophoresis using agarose gel revealed that the smaller DNA restriction fragments that resulted after digestion did penetrate the gel, and a complete cleavage of kDNA by the endonucleases was characterized by a total disappearance of DNA from the top of the gel (Fig. 1). Only *BamH* I proved incapable of digesting all the kDNA of the TP702, TRa, TRa605, TRa606, and TM5 isolates (Fig. 1 B).

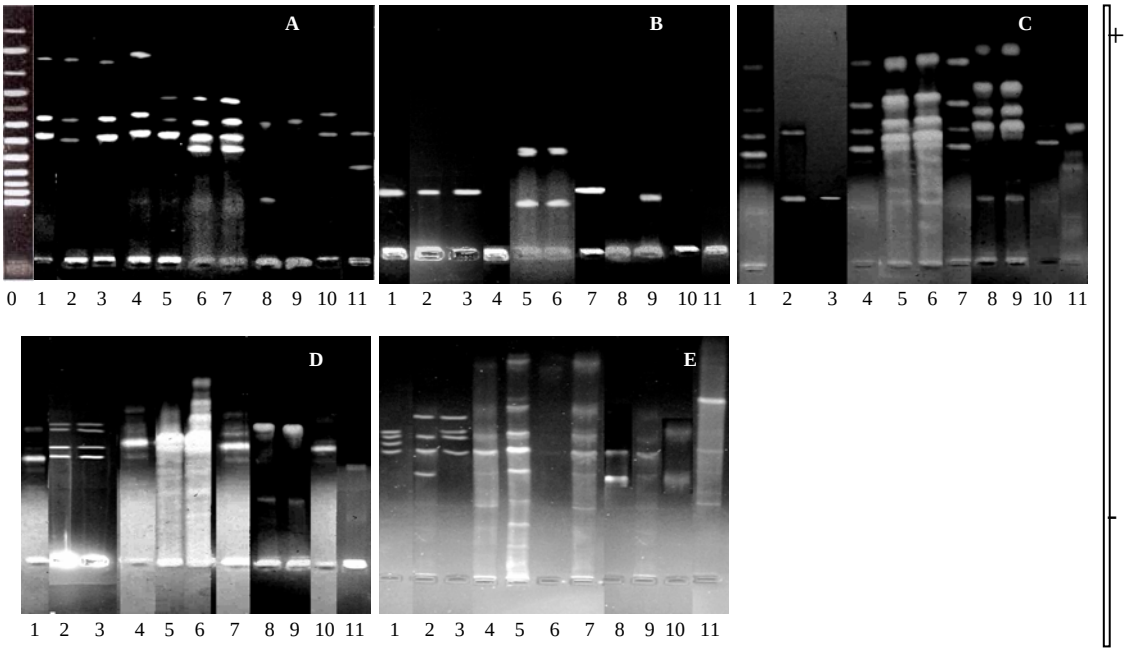

Fig. 1.- Restriction endonuclease analysis of kDNA of *Trypanosoma* isolates. (**A**) kDNA+*EcoR*I; (**B**) kDNA+*Bamh*I; (**C**) kDNAs+*Hae*III; (**D**) kDNAs+*Hinf*I; and (**E**) kDNA+*Msp* I. Lane 1: *T. cruzi* (TC) Maracay strain; Lane 2: *T. cruzi* (TY) strain Y; Lane 3: *T. cruzi* (TCL) strain CL; Lane 4: isolate TP702; Lane 5: isolate TP704; Lane 6: isolate TP706; Lane 7: isolate TP504; Lane 8 *T. rangeli* (TRa, Cajamarca-Peru strain); Lane 9 isolate TRa605; Lane 10 isolate TRa606 and Lane 11 isolate TM5**.** Size markers are 1 Kb DNA ladder fragments (Lane 0).

The three reference stocks (TC, TY, and TCL) had very similar restriction cleavage patterns, with the endonucleasas *EcoR* I, *BamH* I, and *Hinf* I, but were completely different for the enzymes *Hae* III and *Msp* I. The isolates TP702, TP704, TP706, and TP504 had very similar profiles for almost all of the restriction enzymes (Fig. 1, lines 4, 5, 6, and 7) except for the isolate TP706 with the enzyme *Msp* I, where the kDNA was not digested to the same degree as isolate TP702 for the enzyme *BamH* I. The reference stock TRa and the isolated TRa605 and TRa606 had quite similar profiles although completely different from the rest (Fig. 1, lines 8, 9 and 10). The isolate from Mexico, TM5, had a different electrophoretic profile although to a certain extent it can be considered closer to the isolates TRa, TRa605, and TRa606.

When Duplex PCR assay (with T189Fw, Tc189Rv3, TrF3 and TrR8 primers) was used in kDNA samples from the different isolates assayed and of the four reference stocks, it was found that the reference stocks of *T. cruzi* (TCL, TY and TC) presented one amplification product, 100-bp (Fig. 2, lines 9, 10, and 11). The isolates from Peru (TP702, TP704, and TP706) had similar kDNA fingerprints (Fig. 2 lines 3, 4, and 5). The reference stocks characterized as *T. rangeli* presented as an amplification product a band of 500 bp (Fig. 2 line 8), the Peruvian isolates (TRa605 and TRa606) and the isolate from Mexico (TM5) also presented this same band (Fig. 2 lines 6, 7, and 1, respectively), and the isolate TP504 presented two bands as its amplification product, although one of these ha d 100 bp and the other approximately 500 bp (Fig. 2, line 2).

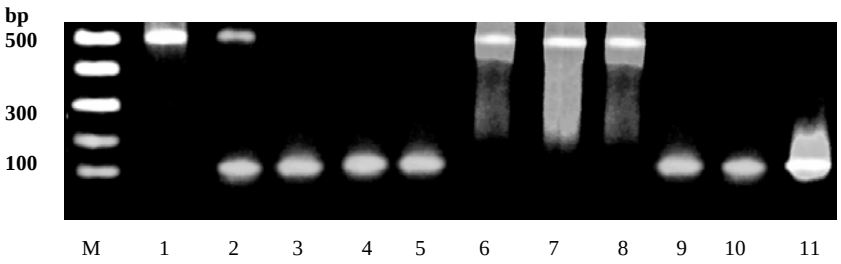

Fig. 2.- Amplification products of the PCR multiplex assay from different *Trypanosoma* strains and isolates, using the primers: T189Fw2, Tc189Rv3, TrF3 and TrR8. In each lane 10 µl of sample were placed. Lane 1: isolate TM5; Lane 2: isolate TP504; Lane 3: isolate TP702; Lane 4: isolate TP704; Lane 5: isolate TP706; Lane 6: isolate TRa605; Lane 7: isolate TRa606; Lane 8: *T. rangeli* (TRa, Cajamarca-Peru strain); Lane 9: *T. cruzi* (TCL) strain CL; Lane 10: *T. cruzi* (TY) strain Y and Lane 11: *T. cruzi* (TC) Maracay strain. Lane M: 100-bp DNA ladder; the lower band shown is 100 bp.

4. Discussion

Many assays have been made with the aim of investigating the correlation between stocks, using markers based on the variation in the restriction pattern of kinetoplast DNA (kDNA). The pattern of the fragments obtained after digestion with restriction endonucleases and gel electrophoresis is usually complex, but it can be specific for groups, species, or even isolates [15], this being known as schizodeme analysis. Mitochondrial DNA has shown the particularity of containing repetitive elements and regions that vary their homology between different species or complexes. Some of these regions are very conserved, while others are variable and differ between the species or isolates [16]. Mitochondrial genes have evolved more rapidly than have nuclear genes, and therefore the rapid evolution of kDNA can be used in the phylogenetic study of the kinetopastid group [17].

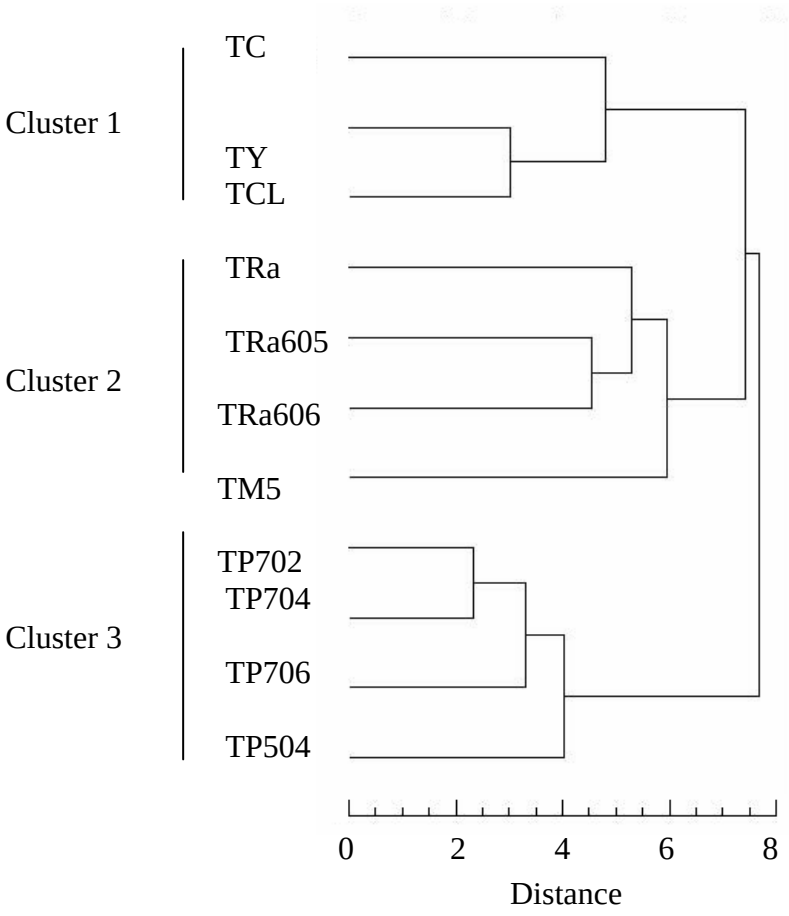

Fig. 3.- Dendrogram based on individual hierarchical cluster analysis (Program Stat-Graphics version 5.0).

In the present study, we obtained the kDNA from seven stocks and from the four reference stocks. This kDNA having been submitted to the action of 5 restriction endonucleases (*EcoR* I, *BamH* I, *Hae* III, *Hinf* I, and *Msp* I). After the statistical analysis of hierarchical cluster grouping by average linkage methods and by using Euclidean distance, made on the basis of the restriction cleavage patterns, the isolates were grouped into 2 clusters. The first cluster was subdivided into four subclusters: subcluster **1**^{a}, containing *T. cruzi* stock Maracay, and the stocks from Peru, TP702 and TP704; subcluster **1**^{b} including *T. cruzi* stock Y and stock CL; subcluster **1**^{c} including stock TP706, phylogenetically very close to *T. cruzi* stock Y and CL, and phylogenetically farther from isolate TP504. This latter forming subcluster **1**^{d}, which come quite close to the second cluster formed by *T. rangeli*, the two isolated from Peru (TRa605 and TRa606), and the isolate from Mexico, TM5.

Also, the kDNA was used for the specific amplification by the PCR technique. Chiurillo et al. [8] had demonstrated that subtelomeric sequences of *T. cruzi* and *T. rangeli* are appropriate for species-specific Duplex PCR detection of these parasites in complex biological samples. The sensitivity reached by duplex PCR is similar to that reported for single *T. rangeli* PCR typing assays with primers targeted at other nuclear targets [18,19]. The duplex PCR assay to detect *T. cruzi* and *T. rangeli* with these primers produced a band of 100 bp with *T. cruzi*, while *T. rangeli* gave a band of some 170 bp and minor high-molecular-weight bands [8]. Our results coincide partially with those of these authors; with the duplex PCR assays , we got a 100 bp band for the three stocks of *T. cruzi* used as a reference and which is common for the stocks isolated from *Triatoma* species of western Peru (TP702, TP704, and TP706). But in the case of *T. rangeli* (TRa) the band that was amplified was 500 bp, slightly greater than the PCR products obtained by the above-mentioned authors. This band coincides with the two stocks isolated from the salivary glands of *Rhodnius ecuadoriensis* (TRa605 and TRa606) and with the isolate of *R. prolixus* TM5 in the Yucatan Peninsula de Mexico. The isolate of *Panstrongylus chinai* (TP504) presented, as the PCR product, two bands, one of 100 bp coinciding with that of *T. cruzi*, and a second band coinciding with the band detected for *T. rangeli*. This suggests that there was a mixture between *T. cruzi* and *T. rangeli*. To test this possibility, we undertook a new cloning, which gave the same result.

In a previous study [5], we characterized these seven stocks and their corresponding reference stocks by lectin agglutination, isoenzyme analysis and the end

products excreted. When these data were statistically treated, together with the data from the present study, we found a grouping of these stocks by heirarchical cluster analysis (or dendogram), as reflected in Figure 3. According to these data, we have three clusters: cluster 1 contains three *T. cruzi* stocks, used as references. Very close to the stocks TY and TCL would be the stocks isolated in the Peruvian Amazon (TP702, TP704, and TP706), which would form cluster 2, and also include stock TP504, although this would be a hybrid between *T. cruzi* and *T. rangeli*. And cluster 3 would be composed of the trypanosoma isolates from salivary glands (TRa, TRa605, TRa606, and TM5), these four stocks being the same *T. rangeli* stock and the phylogenetic separation observed could be due to having been isolates from different hosts and different geographical regions, as happened with the stocks considered to be *T. cruzi* [20]. These findings totally confirm the previous ones obtaines with the same stocks [5]. All these groupings establish a certain correlation between the geographical distribution of the origin of the isolates with the identity of the species.

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DISCUSION.

DISCUSION.

La capacidad para distinguir entre las diferentes especies o cepas, tanto del género *Trypanosoma* como *Leishmania*, es crucial cuando se prescriben tratamientos, así como, cuando se diseñan estrategias de control en los estudios epidemiológicos. Frecuentemente, estas especies, son identificadas en base a su origen geográfico y sobre las manifestaciones clínicas resultantes de las enfermedades. Sin embargo, el origen geográfico es un criterio inadecuado en áreas no endémicas, e igualmente en áreas endémicas donde múltiples especies pueden coexistir. La identificación de las especies infectantes basada en los síntomas clínicos también puede ser problemática, como es el caso de las especies pertenecientes al género *Leishmania*, donde varias especies pueden causar enfermedades, tanto cutáneas como mucocutáneas, mientras otras pueden causar enfermedades viscerales y cutáneas simultáneamente (Schönian y col., 2003).

Es conocido que, las especies del género *Trypanosoma* presentan un pleomorfísmo natural, al cual se ha atribuido la diferencia de severidad con que se presenta la enfermedad en los diferentes hospedadores (Guzmán-Marín y col., 1999). Sin olvidarnos de *T. rangeli*, una especie no patógena para los humanos, pero muy frecuente en infecciones mixtas con *T. cruzi* en algunos países, por lo que, la caracterización de dichas cepas (*T. cruzi* y *T. rangeli*) es crucial para un mejor entendimiento de la interacción entre las diferentes subpoblaciones. Esta información es imprescindible en la clínica de la enfermedad de Chagas (Steindel y col., 1991; Machado y Ayala, 2001).

En los últimos años se ha observado un gran incremento en la incidencia de estas enfermedades, debido principalmente a una expansión geográfica de las mismas, cambios en los hábitos viajeros, migración de

poblaciones, personas no-inmunes moviéndose en regiones endémicas o personas infectadas en regiones no endémicas (Desjeux, 2001).

Por todas estas razones se hace necesaria una correcta caracterización de estas poblaciones de parásitos, lo cual es vital para establecer un mejor diagnóstico, tratamiento, pronóstico y control de estas parasitosis, así como, un mejor entendimiento de la influencia que las variaciones intraespecíficas pueden tener en la epidemiología de estas enfermedades.

En situaciones de campo, el aislamiento y cultivo de tripanosomátidos para su caracterización no siempre es posible, especialmente si encontramos pocos parásitos en las lesiones, sangre, piel y órganos, así como, en los vectores. Los protocolos de aislamiento y cultivo pueden seleccionar clones particulares de poblaciones heterogéneas. Por lo que, lo deseable sería desarrollar herramientas sensibles para identificar y tipificar tripanosomátidos directamente (Breniere y col., 1999).

El objetivo de la presente memoria de tesis doctoral ha sido caracterizar por métodos bioquímicos (aglutinación por lectinas y estudio de isoenzimas), por métodos moleculares (estudio del ADN del kinetoplasto por endonucleasas de restricción y por PCR) y en algunos casos, por las estrategias metabólicas de los tripanosomátidos para degradar los sustratos energéticos (Resonancia Magnética Nuclear de Protones), 25 cepas o especies pertenecientes a los géneros *Trypanosoma* (7 aislados) y *Leishmania* (18 aislados) procedentes de diferentes países: Perú, México y España.

Para prevenir la variabilidad derivada del medio de cultivo, previamente se adaptaron los aislados al mismo medio (Rodríguez y col., 1998). Para lo cual se ensayaron diferentes medios: el más idóneo en el

caso de los aislados del género *Trypanosoma*, fue el medio Grace´s y, para los del género *Leishmania* el medio MTL, ambos adicionados con 10% de suero bovino fetal inactivado; obteniendose una alta densidad de crecimiento en la mayoría de los aislados. Dependiendo de los aislados el crecimiento se encuentra entre el 6,5 x 10^6 – 2 x 10^7 cel/ml.

Una vez determinado el medio más idóneo se procedió a la caracterización de estos aislados, pertenecientes al género *Trypanosoma* y al género *Leishmania*, iniciándose con la aglutinación por lectinas.

Las lectinas se han usado como herramienta en estudios topográficos, y en la distribución y función de los glicoconjugados en los parásitos, debido a sus especificas y bien caracterizadas propiedades de unión a los azucares (Andrade y Saraiva, 1999). Los carbohidratos de la superficie celular de las diferentes especies han sido estudiados por numerosos autores, así como las propiedades de unión a las lectinas (Jacobson, 1994; Jacobson y Doyle, 1996).

En los tripanosomátidos, los carbohidratos de la superficie celular, juegan un papel clave en la entrada del parásito y en su supervivencia dentro de la célula. Los resultados del test de aglutinación muestran que la composición de carbohidratos en las membranas varía de unos aislados a otros.

En el caso de los siete aislados del género *Trypanosoma* más las cuatro cepas de referencia, todos aglutinaron con la lectina Con A. Indicando que todos tienen moléculas de α-D-glucosa y α-D-manosa en sus membranas, aunque en los aislados TP504, TP702 y TP706 esas moléculas son más numerosa que en los otros. Con la lectina de *Vicia villosa* solo aglutinaron los aislados de las glándulas salivares de *R. ecuadoriensis* (TRa605 y TRa606) y la cepa de referencia TRa, indicando la presencia de

N-acetil-galactosamina en sus membranas, estos datos coinciden con los obtenidos por otros autores (Acosta y col., 1991), quienes determinaron que la lectina VV reacciona selectivamente solo con las cepas de *T. rangeli*.

Con la lectina de germen de trigo (WGA) se observa un patrón de aglutinación heterogéneo en los diferentes aislados siendo muy variable la cantidad de residuos N-acetil-D-glucosamina en sus superficies. La lectina PNA claramente separa la cepa de referencia TC del resto de los aislados de Perú y México. Diferentes autores han encontrado que cepas de Venezuela y Brasil, incluidas en el tipo 2, son tipo PNA, que como con nuestra cepa de referencia TC no aglutinan con la lectina de *Arachis hypogaea* y si con la lectina WGA (Vivas y col., 1979; Schottelius, 1982). El aislado de México (TM5) presenta una fuerte aglutinación con la lectina PNA, distinguiéndolo de los aislados peruvianos, demostrando que existe ácido neuramínico en abundancia en la superficie de los epimastigotes de estos aislados.

Para el caso de los aislados del género *Leishmania* todos los aislados aglutinan con la lectina Con A, los resultados coinciden con los de Dwyer (1977), todas las cepas tienen en sus membranas moléculas de α-D-glucosa y α-D-manosa, aunque en diferente proporción. La aglutinación de WGA para *L.* (*L.*) *donovani* reportada por Bandyopadhyay y col. (1991) no se ha observado en este estudio ni en otros (Sacks y col., 1995; Andrade y Saraiva, 1999), aunque si se ha observado aglutinación con los aislados españoles, así como con *L. (L.) peruviana* y otros aislados de Perú, LP4, LP5, LP7, LP8, LP9, LP10, LP12, LP13 y LP14, estos aislados poseen N-acetil-D-glucosamina en sus membranas. Todos los aislados aglutinan con la lectina PNA, demostrando la presencia de ácido neurámínico en sus membranas. Los resultados obtenidos coinciden con todos los reportados por diferentes autores (Shottelius, 1982; Andrade y Saraiva, 1999). Para el

caso de la lectina VV solo los aislados de Perú aglutinan, a excepción de LP4.

Los miembros de la familia Trypanosomatidae son incapaces de degradar completamente los carbohidratos, incluso en presencia de oxigeno, produciendo CO_2 y ácidos dicarboxílicos (Sánchez-Moreno y col., 1995). La proporción relativa de esos productos varía de unas subespecies a otras. Por espectroscopia de RMN hemos identificado los metabolitos que excretan los aislados en el medio de cultivo. En el caso de los tripanosomas todos los aislados excretan succinato, acetato y alanina. Y para los aislados de *Leishmania* excretan piruvato y acetato en mayor proporción y succinato y alanina en menor proporción. La proporción relativa de los productos varía entre las subespecies.

La electroforesis de isoenzimas es uno de los métodos empleados para detectar diferencias entre enzimas con propiedades catalíticas similares pero con distinta estructura molecular, lo que permite agrupar cepas de acuerdo a sus perfiles isoenzimáticos idénticos.

Los patrones isoenzimáticos de algunos de los aislados estudiados nos permiten, a simple vista, observar variaciones en cuanto al número de bandas y sus puntos isoeléctricos. En el caso de los tripanosomas los 3 aislados de las glándulas salivales de *R. ecuadoriensis* son los más homogéneos para los 6 sistemas enzimáticos (GPI, IDH, MDH, ME, PGM, SOD). La cepa TC muestra diferencias en el loci de los 6 sistemas enzimáticos con respecto al resto de aislados de Perú y México. Los aislados de Perú (TP702, TP704 y TP706) muestran similaridad en los loci enzimáticos GPI, MDH, ME, PGM y SOD. Comparando nuestros resultados con los de Ebert (1982), encontramos grandes coincidencias en los perfiles de algunos de las enzimas ensayadas (PGM, MDH y ME),

concluyendo que los aislados peruvianos pueden ser correlacionados con los aislados de *T. cruzi* incluidos en el zimodema Z1 por este autor y confirmado recientemente por Sousa y col. (2006).

En el caso de los aislados del género *Leishmania* también se han usado estos 6 sistemas enzimáticos GPI, IDH, MDH, ME, PGM y SOD para las cepas *L. (V.) peruviana*, *L. (L.) donovani*, *L. (L.) infantumI*, *L. (L.) infantumII*, LS1, LP1, LP2, LP3, LP4, LM1, LM2 y LM3. En esta ocasión incluimos el sistema enzimático G6PDH para las cepas *L. (V.) braziliensis*, *L.(L.) amazonensis* y LP5 - LP14, que en un estudio anterior nos permitió observar diferencias entre especies (Miralles y col., 2002). Estos siete sistemas enzimáticos pueden identificar parásitos del género *Leishmania* (Shamsuzzaman y col., 2000).

L. (L.) donovani y *L. (V.) braziliensis* presentan un perfil diferente del resto de los aislados. *L. (V.) peruviana* difiere de los otros aislados procedentes de Perú, no obstante los aislados LP2 y LP3 muestran perfiles muy semejantes con las enzimas IDH, ME y SOD. *L. (L.) amazonensis* solamente se diferencia del resto de los nuevos aislados con las enzimas IDH y MDH, aunque el nuevo aislado LP11 presenta un perfil semejante para el enzima MDH con *L. (L.) amazonensis*, este mismo aislado presenta grandes diferencias con respecto a los otros aislado en las enzimas IDH y SOD. Los aislados LP6, LP13, y LP14 comparten el mismo perfil que *L. (L.) amazonensis* para el enzima málico. Los aislados LP6, LP9, LP11 y LP12 con la enzima G6PDH se diferencian claramente de las 2 cepas de referencia y del resto de los aislados. La enzima PGM no permite detectar ninguna diferencia significativa entre todos los aislados objeto de estudio.

Son numerosos los ensayos que se han realizado con el fin de investigar la correlación entre cepas, utilizando marcadores con base en la

variación del patrón de restricción del ADN del kinetoplasto o mitocondrial (ADNk). El patrón de los fragmentos obtenidos tras la digestión con endonucleasas de restricción y electroforesis en gel usualmente es complicado, pero puede ser específico para grupos, especies o aún específico de aislados (García y Mendoza-León, 2000), es lo que se conoce como análisis de schizodemo. El ADN mitocondrial ha mostrado la particularidad de contener elementos repetitivos y regiones que varían su homología entre las diferentes especies o complejos; algunas de las regiones están muy conservadas, mientras que otras son variables y diferentes entre las especies o aislados (Hide y Tait, 1991). Los genes mitocondriales evolucionan más rápido que los genes nucleares, por lo tanto, es posible utilizar la rápida evolución del ADNk en el estudio filogenético del grupo de los Kinetoplastidos (McManus y Bowles, 1996).

En este estudio se ha obtenido el ADNk de todos los aislados tanto del género *Trypanosoma* como los pertenecientes al género *Leishmania*, este ADNk ha sido sometido a la acción de 5 endonucleasas de restricción (*EcoR* I, *BamH* I, *Hae* III, *Hinf* I y *Msp* I).

La electroforesis usando geles de azarosa, revela pequeños fragmentos ADNk resultantes de la digestión de las endonucleasas. Todas las enzimas, ha excepción de la enzima *BamH* I, digieren completamente el ADNk de los 7 aislados y de las 4 cepas de referencia de *Trypanosoma*. La enzima *BamH* I es incapaz de digerir el ADNk del aislado TP702, TRa, TRa605, TRa606 y del aislado de México TM5.

Las 3 cepas de referencia de tripanosoma (TC, TY y TCL) presentan un perfil de restricción muy semejante con la endonucleasas *EcoR I*, *BamH I* y *Hinf I*, pero muy diferente para las enzimas *Hae III* y *Msp I*. El grupo de aislados TP702, TP704, TP706 y TP504 presentan un perfil electroforético

muy similar, excepto el aislado TP706, su ADN no es digerido por el enzima *Msp* I. El aislado de referencia TRa y las cepas TRa605 y TRa606 tienen perfiles muy semejante entre si pero completamente distintos al resto de aislados y con todas las endonucleasas, el aislado TM5 tiene una gran similitud con estos últimos aislados.

Al igual que sucedía en el caso de los aislados de *Trypanosoma*, el ADN del kinetoplasto de algunos de los aislados del género *Leishmania* (*L*. (*V*.) *braziliensis*, *L*. (*L*.) *amazonensis* y los aislados LP6 y LP11) no son digeridos por la endonucleasas *BamH* I y *Hinf* I. *L*. (*V*.) *braziliensis* y *L*. (*L*.) *amazonensis* se diferencian claramente entre si y con respecto al resto de aislados con las 5 enzimas de restricción. Los aislados desde el LP1 al LP10 muestran un perfil de restricción muy semejante con la mayoría de las endonucleasas ensayadas. El aislado LP11 tiene un patrón electroforético completamente diferente al resto de los aislados y los aislados LP12, LP13 y LP14 son muy homogéneos entre si pero diferentes al resto.

El ADNk también se ha utilizado para su amplificación específica por la técnica de PCR. Chiurillo y col. (2003) habían demostrado que las secuencias subteloméricas de *T. cruzi* y *T. rangeli* son específicas de especies, detectando a esos parásitos en complejas muestras biológicas por PCR múltiple. La sensibilidad de la PCR múltiple es similar a la descrita por Souto y col. (1999) y Vargas y col. (2000), en ensayos con *T. rangeli* utilizando PCR tradicional con cebadores de secuencias nucleares dianas. Con la PCR múltiple se detecta *T. cruzi* y *T. rangeli,* obteniéndose con esos cebadores una banda de 100-pb para *T. cruzi*, mientras que con *T. rangeli* se obtiene una banda de unos 170-pb y bandas de menor peso molecular (Chiurillo y col., 2003).

Nuestros resultados son parcialmente coincidentes con los de estos autores, con los ensayos con PCR múltiple obtenemos una banda de 100-pb para las tres cepas de *T. cruzi* usadas como referencia y que es común para las cepas aisladas de especies de *Triatoma* en el oeste del Perú (TP702, TP704 y TP706). Pero en el caso de *T. rangeli* (TRa) la banda que se amplifica es de 370-400-pb, superior a los productos de la PCR obtenidos por los autores anteriormente mencionados, esta banda es coincidente con las dos cepas aisladas de las glándulas salivales de *Rhodnius ecuadoriensis* (TRa605 y TRa606) y con el aislado de *R. prolixus* TM5 en el Yucatán, Península de México.

El aislado de *Panstrongylus chinai* (TP504) presenta como producto de la PCR dos bandas, una de 100-pb coincidente con la de *T. cruzi* y una segunda banda coincidente con la banda detectada para *T. rangeli*. Esto nos podría llevar a pensar que ha habido una mezcla entre *T. cruzi* y *T. rangeli*. Por lo que, para descartar esta posibilidad se procedió de nuevo a su clonaje, manteniéndose el mismo resultado.

Para el caso de *Leishmania* se ha desarrollado una PCR múltiple que utiliza un único cebador 5', conservado en todas las especies de *Leishmania* y 3 cebadores con diferentes secuencias en 3', cada uno de ellos especifico de los diferentes complejos (Harris y col. 1998). La identificación de los complejos *Leishmania* está basada en el tamaño de los productos de ambos así como en la secuencia específica de los cebadores. Para *L.* (*L.*) *donovani*, el producto obtenido tiene un tamaño que va desde 360 a 370 pb al igual que el aislado LP7; para las tres cepas aisladas de México (LM1, LM2 y LM3), los productos tienen un tamaño de 270 a 280 pb, la cepa de referencia amplifica una banda de 260 a 270 pb; y para *L.* (*V.*) *braziliensis* y los aislados LP1 al LP6 y LP8 al LP14, los productos tienen un tamaño de 150 a 170 pb.

Se esperaba que la PCR fuera específica solo para las cepas pertenecientes a los complejos *L.* (*L.*) *donovani*, *L.* (*V.*) *braziliensis*, y *L.* (*L.*) *mexicana*; sin embargo, al evaluar la PCR con las cepas *L.* (*L.*) *infantum* I y II y el aislado denominado por nosotros como LS1 amplificaba una banda común a los tres de 470 a 490 pb. Probablemente, este comportamiento se deba a que las regiones amplificadas sean altamente conservadas en todas las especies, con algunas divergencias, favoreciendo la hibridación de los oligos (Caceres y Montoya, 2002).

La identificación más apropiada y su relación filogenética se obtiene con el análisis euclideo global de todos los datos obtenidos en las cuatro publicaciones que conforman esta memoria de tesis.

Para el caso de los tripanosomas, tendríamos tres cluster: En el **cluster 1** estarían las tres cepas de *T. cruzi* utilizadas como referencias, muy próximas a las cepas TY y TCL estarían los aislados en la amazonía de Perú (TP702, TP704 y TP706) y que formarían el **cluster 2**, en el cual también incluiríamos al aislado TP504, aunque este sería como un híbrido entre *T. cruzi* y *T. rangeli*. El **cluster 3** estaría formado por los tripanosomas aislados de las glándulas salivales de *Rhodnius* sp. (TRa605, TRa606 y TM5), estos tres aislados serían la misma cepa *T. rangeli* (Grisard y col., 1999) y la separación filogenético observada podría deberse a haber sido aislados de diferente hospedador y de diferente región geográfica, al igual que sucede con los aislados considerados como *T. cruzi* (Moreno y col., 2002).

Todos los grupos establecidos muestran una cierta correlación entre la distribución geográfica, el origen de los aislados y la identificación de las especies.

En el caso de las leishmanias tras realizar el análisis estadístico correspondiente, resultan 5 grupos. En el primer grupo (**cluster 1**) tendríamos a *L.* (*L.*) *donovani* junto a los aislados LP4 y LP7 que formarían el complejo *donovani*, seguramente se tratarían de especies pertenecientes a la forma visceral del nuevo mundo (*L.* (*L.*) *chagasi*); el **cluster 2** estaría formado por los aislados LP1 a LP3, LP6 y LP9 a LP11; filogenéticamente intermedios entre el complejo *donovani* (**cluster 1**) y el complejo *braziliensis* (**cluster 3**) o bien pudieran tratarse de híbridos, lo que es conocido en el continente americano (Hernández y col., 1991), esto no es una sorpresa ya que esos aislados son de la misma área geográfica y estaban presentes en lesiones cutáneas crónicas similares, comprometiendo las mucosas, y la cepa *L.* (*L.*) *donovani* está filogenéticamente próxima a las cepas peruvianas (Rodríguez-González y col., 2006).

El **cluster 3** se podría dividir en dos subgrupos uno formado por *L.* (*V.*) *peruviana*, junto a los aislados LP5 y LP14, y un segundo subgrupo formado por *L.* (*V.*) *braziliensis* y el aislado LP8, estos dos subgrupos formarían parte del complejo *braziliensis* (Laison y Saw, 1987); las tres cepas de *L.* (*L.*) *infantum* (*L.* (*L.*) *infantum* I y II y la LSI) formarían el **cluster 4** y en el **cluster 5** incluiríamos a las tres cepas mexicanas (LM1,LM2 y LM3) formando un subgrupo y en otro subgrupo tendríamos a *L.*(*L.*) *amazonensis*, estos dos subgrupos formarían el complejo *mexicana*. Se observa una correlación entre la distribución geográfica y la identificación de las especies.

Las especies de Leishmania del Nuevo Mundo de los diferentes complejos se pueden encontrar en el mismo tipo de muestras clínicas, por ejemplo, *L.(L.) mexicana* y *L. (L.) chagasi* se han encontrado en nódulos cutáneos (Neva y col., 1997), *L.(V.) braziliensis* y *L.(L.) amazonensis* han sido encontradas también en lesiones cutáneas (Grimaldi y Tesh, 1993),

entre otras. Las distintas especies de *Leishmania* son indistinguibles morfológicamente pero se pueden distinguir en la forma que cursan, por lo que la taxonomía de este género reviste una especial importancia médica. Es muy difícil establecer el limite de especies existentes en Perú por el continuo hallazgo de nueva especies y en estos últimos años de formas hibridas (Mita, 2001; Flores y col., 2002). Además, la cercanía con Brasil, Colombia, Chile, Bolivia y Ecuador ocasiona una mezcla de especies, motivada principalmente por las migraciones de humanos pero mucho mas por las de los animales que se desplazan por la amazonía, trayendo parásitos antes desconocidos en estas zonas.

La necesidad de diferenciar y caracterizar las poblaciones de los parásitos, surge para establecer un mejor diagnóstico, tratamiento, pronóstico, control e influencia que las variaciones intraespecíficas pueden tener en la epidemiología de estas enfermedades.

Por medio de las técnicas empleadas en este estudio se ha podido completar el estudio filogenético así como identificar de manera rápida los aislados tanto del género *Trypanosoma* como de *Leishmania* para propósitos epidemiológicos en áreas donde la enfermedad de Chagas o la leishmaniasis respectivamente son endémicas.

CONCLUSIONES.

CONCLUSIONES.

1. Se han caracterizado 25 aislados: 7 pertenecientes al género *Trypanosoma* y 18 al género *Leishmania*, procedentes de diferentes países: Perú, México y España, por técnicas Bioquímicas y de Biología molecular.

2. Gracias a las técnicas empleadas hemos aportado nuevos datos en la epidemiología de la enfermedad de Chagas y de la Leishmaniasis.

3. El medio de cultivo óptimo para el crecimiento de los nuevos aislados es el medio Grace´s para los pertenecientes al género *Trypanosoma* y, el medio MTL para los del género *Leishmania*.

4. Mediante la técnica de aglutinación por lectinas se ha puesto de manifiesto que la composición de los carbohidratos de superficie celular de los parásitos varían de unos aislados a otros. Permitiéndonos distinguir a los aislados TRa605 y TRa606 del género *Trypanosoma* como pertenecientes a *T. rangeli*.

Sin embargo, esta técnica en el caso de los aislados del género *Leishmania* no nos permite diferenciarlos claramente, aunque si se observan diferencias cuantitativas a nivel de los carbohidratos presentes en las membranas.

5. Nuevamente, por el análisis isoenzimatico, los aislados TRa605 y TRa606 junto con la cepa de referencia *T. rangeli* son muy homogéneos en sus perfiles isoenzimáticos.

Los aislados peruanos pertenecientes al género *Trypanosoma* están correlacionados con *T. cruzi* incluido en el zimodema Z1.

Los 7 sistemas enzimáticos utilizados nos permiten distinguir los aislados del género *Leishmania* aunque no es capaz de establecer grupos.

6. El grupo de aislados TP702, TP704, TP706 y TP504 presentan un perfil electroforético muy homogéneo cuando su ADNk es sometido a la acción de 5 endonucleasas de restricción, y muy similar al de las 3 cepas de referencia pertenecientes al género *Trypanosoma*.

T. rangeli, TRa605, TRa606 y TM5 son muy semejantes entre si y completamente distintos al resto de los aislados del género *Trypanosoma*.

La digestión del ADNk de los aislados del género *Leishmania* los separa en dos grupos: El primer grupo formado por LP1 a LP10 y el segundo grupo formado por LP12 a LP14. Mientras que el perfil del aislado LP11 es totalmente diferente a ambos.

7. Mediante la técnica de PCR los aislados del género *Trypanosoma*: TP702, TP704 y TP706, se agrupan con las 3 cepas de referencia de *T. cruzi.*

Los aislados TRa605, TRa606 y TM5 se agrupan con la cepa de referencia *T. rangeli*. Mientras que, TP504 parece ser un hibrido entre *T. cruzi* y *T. rangeli*.

En el caso de los aislados del género *Leishmania*, la PCR múltiple si nos ha permitido agruparlos claramente en 4 grupos. Grupo 1 formado por la cepa de referencia *L.(L.) donovani* junto con el aislado LP7. El grupo 2 compuesto por los aislados procedentes de México. Los aislados LP1 a LP6 y LP8 a LP14 junto con *L. (V.) braziliensis* conformando el grupo 3. El grupo 4 incluye los aislados de España.

CONCLUSION GENERAL

La identificación y la relación filogenética entre los aislados estudiados en la presente memoria se establece con el análisis estadístico global de todos los resultados obtenidos con todas las técnicas. Los aislados procedentes de la amazonía de Perú: TP702, TP704 y TP706 formaría un cluster muy próximo filogenéticamente a las cepas de referencia de *T. cruzi*. Los aislados de las glándulas salivales de *Rhodnius* sp.: TRa605, TRa606 y TM5, son *T. rangeli*. El aislado TP504 es un hibrido entre *T cruzi* y *T. rangeli.*

En el caso de los aislados del género *Leishmania* y por el mismo análisis estadístico resultan 5 grupos: el complejo *donovani* que estaría formado por *L.(L.) donovani* y los aislados LP4 y LP7, los cuales son probablemente especies pertenecientes a la forma visceral del Nuevo Mundo (*L.(L.) chagasi*). Los aislados LP1 a LP3, LP6 y LP9 a LP11, son filogenéticamente intermedios entre el complejo *donovani* y el complejo *braziliensis*. Los aislados LP5, LP8 y LP14 son incluidos en el complejo *braziliensis* junto con *L. (V.) peruviana* y *L. (V.) braziliensis*. Los aislados procedentes de España pertenecen a *L.(L.) infantum*. Y los 3 aislados mexicanos se relacionarían con *L. (L.) amazonensis* formando el complejo *mexicana*. Observándose una gran correlación entre la distribución geografía y la identificación de los diferentes aislados.

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