CHAPTER 1

A GUIDE FOR THE CUTANEOUS LEISHMANIASIS CONNOISSEUR

Alexis Mendoza-León, Jeffrey J. Shaw and Felix J. Tapia

Leishmania parasites are named after W. B. Leishman,[1] who first associated the organisms with the initial symptoms of the disease. The disease, termed leishmaniasis or leishmaniosis,[2] is an example of a zoonosis that reaches man through an insect. The causative agents are different species of the parasite, which are transmitted by sandfly of the *Phlebotomus* (Old World) and *Lutzomyia* (New World) genera.

Leishmaniasis is not a single entity but comprises an assortment of syndromes with characteristic clinical, histopathological and immunological features. The World Health Organization (WHO) has estimated that about three million human beings suffer the diseases, 12 million are infected, and 350 million live in risk areas.[3]

WHO USES *LEISHMANIA* AND WHY?

Many species of Leishmanias are easily handled both in vivo and in vitro in the laboratory. This has resulted in them being used as research tools in a wide range of the basic biological sciences. In the field of epidemiology they have been studied both qualitatively and quantitatively and have been important in elaborating control measures and mathematical models of vector born zoonotic diseases. Their intracellular habitat has been particularly important in attracting immunologists interested in the disease process associated with organisms that live in macrophages, and in the 1960s studies concentrated on *L.(Leishmania) enriettii* in guinea pigs. As the genetic susceptibility of different strains of mice became defined, however, studies moved to species such as *L.(L.) donovani* and *L.(L.) major* that infect mice. Immunologists also use the mouse model to define the subsets of lymphocytes and their products involved in intracellular infections. Geneticists use *Leishmania* parasites to study the molecular processes associated with susceptibility, such as Nramp gene in both mice and humans.

Molecular and Immune Mechanisms in the Pathogenesis of Cutaneous Leishmaniasis, edited by Felix J. Tapia, Gisela Cáceres-Dittmar and Martín A. Sánchez.

A number of *Leishmania* species have been used to study the role of the cell membrane in infection and the genes associated with its different structures. These have highlighted the importance of a series of surface components, such as ectoenzymes (gp63), lipophosphoglycan (LPG) and glyosylphosphatidylinositols (GIPLs) in the infective process. Molecular biologists have also used *Leishmania* to study the underlying processes involved in both kinetoplastic DNA (kDaNA) and nuclear DNA (nDNA) replication. These studies have coincidentally led to a better understanding of the taxonomy of the *Leishmania* and to the development of new diagnostic methods. Leishmanias are also of interest to geneticists and taxonomists interested in the population dynamics and taxonomy of protozoa that reproduce principally asexually.

The use of *Leishmania* in different fields of the basic sciences continues to grow. Besides this, however, an increasing number of research workers are using the ever expanding arsenal of molecular methods to ask questions about the parasite itself. It is sometimes difficult to draw the line between these two groups.

THE PARASITE

Order Kinetoplastida

The genus *Leishmania* belongs to the Subkingdom Protozoa, Phylum Sarcomastigophora, Order Kinetoplastida and Family Trypanosomatidae (Fig. 1.1), which also includes the genus *Trypanosoma* that comprises several other human pathogens. The Kinetoplastidae are unicellular elongated or spherical organisms that possess a single flagellum at the anterior end, and have a characteristic structure called the kinetoplast that extends to a single large mitochondrion.

Morphology

The life cycle of *Leishmania sp.* is commonly and erroneously viewed as consisting simply of amastigotes in mammals and promastigotes in sandfly.[4] The parasites exist in various morphological forms: promastigotes and paramastigotes in the vector, and amastigotes in macrophages or other antigen-presenting cells of the mammalian host. The forms are named by the arrangement of the flagellum, being amastigotes spherical organisms with no external flagellum, promastigotes those with an antinuclear kinetoplast and a flagellum emerging from the anterior end of the body, and paramastigotes characterized by a paranuclear kinetoplast and a flagellum emerging from the anterior end.[4]

Leishmania Life Cycle in the Invertebrate Host

Most ingested *Leishmania* amastigotes are destroyed within the first hours, and those surviving transform into promastigotes that multiply rapidly in the blood meal. The promastigotes remain in the digestive tract without penetrating the hemocoele. After a few days, numerous

procyclic promastigotes conquer the gut of the insect (see chapter 2). Thin and elongated procyclic promastigotes (12 μm in body length) attach to the midgut epithelium by inserting their long flagella between the microvilli that line the midgut.[4] They migrate to the cardiac valve, where they transform into short, spherical, non-dividing promastigotes (<12 μm in body length) that attach to the cuticular surface of the stomodeal valve by a modified flagellum. The parasites are released from the midgut and penetrate the pharynx (prosboscis) as metacyclic promastigotes, also termed paramastigotes.[4] Originally, these forms were thought to be opisthomastigotes, which are characterized by a postnuclear kinetoplast, but the term paramastigote was

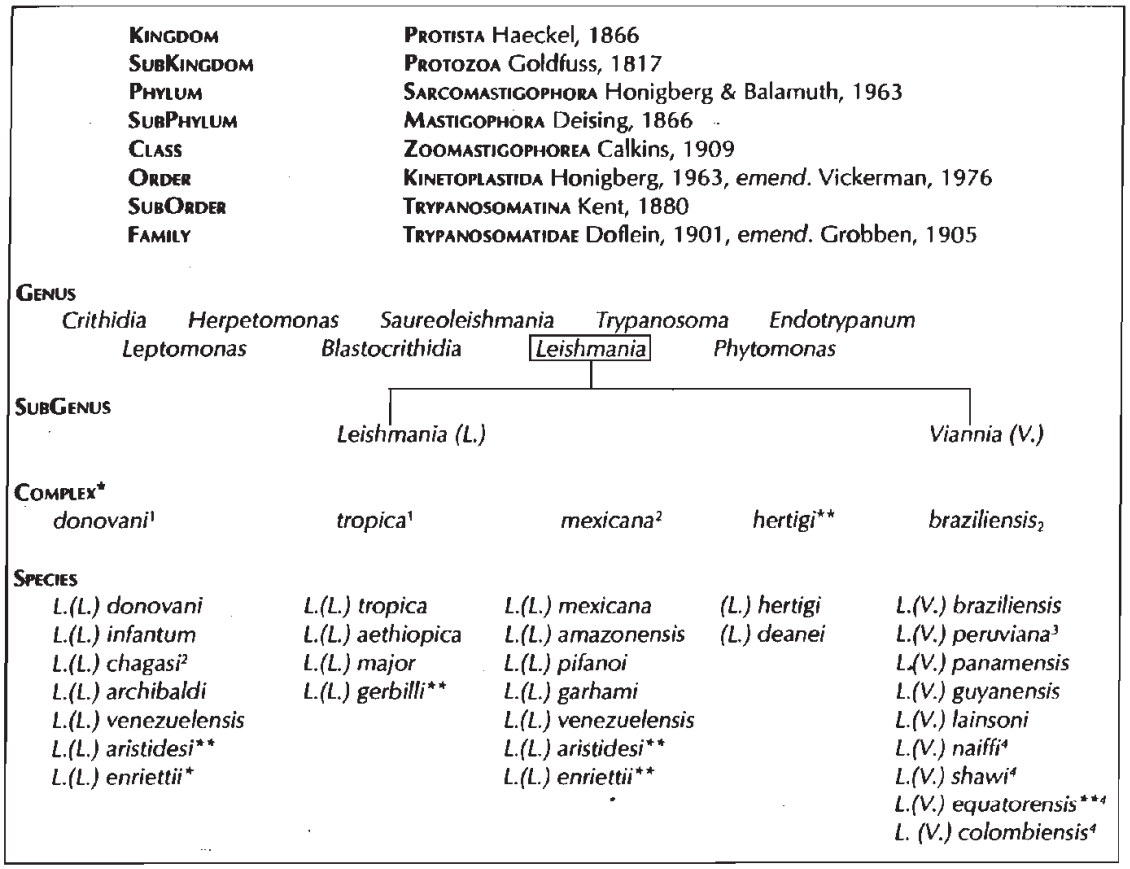

Fig. 1.1. The systemic position and classification of the genus Leishmania. *The classification follows that used by the WHO Technical Report (1990)[67] and Lainson and Shaw (1987).[11] 1. Old World* Leishmania. *2. New World* Leishmania. *3. It is undistinguished from* L. (V.) braziliensis *by RFLP analysis.[103] 4. They have been recognized as new species in the subgenus Viannia.[99-102]*

* *term without a taxonomic status.*

** *not recorded in man.*

introduced by Janovy et al[5] to describe spherical or oval parasites with the kinetoplast lying beside or just posterior to the nucleus.[5] From the prosboscis the metacyclic promastigotes are ousted to the new mammalian host.

The metacyclic promastigotes have been associated with infectivity, loss of the binding receptor for peanut agglutinin (PNA), and structural modifications of LPG[6] (see chapter 2).

Leishmania Life Cycle in the Vertebrate Host

The infected sandfly may inoculate about 10-200 promastigotes into the dermis, where they activate complement and bind CR3 (see chapter 11). Most free promastigotes will be destroyed by polymorphonuclear leukocytes and eosinophils, but some attach to receptors on the surface of dermal macrophages and are phagocytosed (see chapter 2). Within the macrophage, the promastigotes rapidly transform into amastigotes and move to the parasitophorous vacuoles or phagolysosomes, where they may resist destruction and readily multiply. Amastigote-filled macrophages burst, and the parasites reinfect other cells.[7]

Besides macrophages, cutaneous Langerhans cells may get infected by *Leishmania* parasites.[8] These dendritic cells are the main antigen-presenting cells of the skin, which after priming migrate to the regional lymph node where they stimulate naive T cells into memory T cells.[9] Recently, it has been shown that lymph node dendritic cells harbor the parasites for prolonged periods, thus contributing to the maintenance of protective immunity.[10] In addition, dendritic cells may act as foci of reinfection under conditions of immunosuppression.

Taxonomy, Systematic Position and Classification of *Leishmania*

Historical background about the *Leishmania* classification has previously been described.[11] The two proposed subgenera are based on the distribution of the parasites in the intestine of the sandfly vectors. In the subgenus *Leishmania*, the promastigotes develop in the midgut and foregut of the insect (Section Suprapylaria), whereas in the subgenus *Viannia* parasites are restricted to the hindgut (Section Peripylaria).[11]

Pifano was the first to group the New World parasites into two complexes: *brazilienzis* and *pifanoi*,[12] based on the description by Medina and Romero.[13] Afterward, Lainson and Shaw categorized these parasites in the *braziliensis* and *mexicana* complexes, based on the behavior of the parasites in the sandfly gut.[14-18] This classification produced trinomial subspecific names, such as *L. brazilienzis guyanensis* or *L. mexicana venezuelensis*, which were cumbersome and misleading. Finally, Lainson and Shaw[11] revised the classification, and raised the subspecific names to specific taxonomic levels of subgenus and species, and extended its application to Old World parasites (Fig. 1.1).

The division into subgenera is accepted and supported by both biochemical and molecular results. The use of subgeneric names is not obligatory, as is the use of genus and species, but it is useful and advisable as it draws attention to the significant differences between these two groups of parasites that is often not generally appreciated. Subgeneric names are used more by those interested in taxonomy than those who use *Leishmania* as immunological, biochemical or molecular models. The concept of the different complexes is more controversial and evidence is accumulating that supports more divisions at this level. The term complex has no taxonomic status, and is an informal term used to show relationships below the subgeneric level. Remember that the family Trypanosomatidae is a group of morphologically similar parasites that are incredibly diverse physiologically and molecularly. Thus, differences in the ribosomal gene sequences between the genera *Leishmania* and *Trypanosoma* are of the same order as those between mammals and amphibia.[19] Such differences are only apparent, however, when the correct analytical methods are used for homologous characters.

LEISHMANIA SPECIES–IDENTIFICATION AND CLASSIFICATION

Although identification and classification are related, they are different. It may be possible to identify and differentiate an isolate from others but it may not be possible to classify it. To classify a *Leishmania* one needs to compare characters with those of other known taxa and determine the degree of similarity. The theoretical approaches to classifying *Leishmania* vary according to the type characters and during the past 20 years[20] three different approaches have been used: Eclectics or Evolutionary Taxonomy, Phenetics (Numerical Taxonomy) and Cladistics. When homologous characters are used the results are similar but the results may be different when they are not. Today the cladistical approach is preferred since it follows the principle that speciation is a dichotomous process and that species at nodes become extinct. The method uses the principle of maximally parsimonious trees or the tree with the least branches.

So far cladograms for *Leishmania* species have been based on isoenzyme results.[21,22] A phenetic tree based on the Intergenic Region Typing (ITS) fragments of *Viannia* strains was similar to the isoenzyme tree mentioned above, but a few strains, identified by isoenzymes as *L.(Viannia) braziliensis*, clustered with those of other species.[23] Enzymes are phenotypic characters and the same electrophoretic mobility may be associated with molecules whose base pair sequences vary as much as 30%. Eventually trees based on homologous gene sequences may replace those derived from phenotypic characters, but it is likely that the major divisions will remain unaltered.

The species concept for *Leishmania*, which are predominantly asexually reproducing organisms, approaches that used by bacteriologists. A new species must be described using as many phenotypic and

genotypic characters as possible. The decision, however, as to whether or not a parasite is given a new specific name is the responsibility of the investigator(s) and there are presently no set rules concerning this. When examined phenetically Nei's genetic difference between most of the named species for enzymes is in the order of 0.5 to 0.6.[22]

The enzootic cycle, in a given geographical area, of most *Leishmania* species involves one mammal and one sandfly species. These are respectively the major reservoir and vector. These two environments are the principal selective pressures that will be modified if the sandfly alters its feeding habits. The continuously changing interplay of vector/reservoir that occurs in nature may well be reflected in the genomic plasticity which seems to be a major character of the leishmanial genome. However, there is evidence that one gene pool is selected by a specific reservoir/vector relationship and when this happens we have a species which is genetically homogenous. Leishmanial speciation is almost certainly due to parasites becoming adapted to new hosts and vectors. It is presently unknown whether one may be more important than the other.

THE MOLECULAR BIOLOGY OF THE PARASITE

Genome Organization

As in other eukaryotic cells, the genome of *Leishmania* consists of at least three types of DNA in terms of autonomous replication: nuclear DNA, mitochondrial DNA referred as kinetoplast DNA, and extrachromosomal amplified circular DNA. The latter have been related to drug resistance,[24] but the extrachromosomal particle CD1, described in *L. (L.) donovani* and *L. (L.) mexicana* is of unknown function.[25]

In addition, virus-like DNA particles have also been described in *Leishmania* parasites.[26] Although their biological functions are unknown they may be very useful in transfection experiments for the study of genetic expression.[27,28]

DNA Content and Karyotype

The information available from kinetic analysis shows that the haploid genome of *Leishmania* has a size of 3.3-6.5 x 10^7 bp (≈0.116 pg DNA/cell) and is distributed over several chromosomes.[29,30] Although there is no evidence available for typical mitotic chromosome condensation, a more comprehensive view of the genomic organization of *Leishmania* and other parasites has been obtained using Pulse Field Gel Electrophoresis (PFGE) and variations of this technique like the Orthogonal Field Alternate Gel Electrophoresis (OFAGE) and Clamped Homogeneous Electric Field Electrophoresis (CHEF).[31] This high resolution methodology has allowed the isolation of DNA fragments ranging in length from several thousand to several million base pairs.

In both New and Old World *Leishmania* parasites, different species exhibit a karyotype of 20-25 chromosome-size DNA bands. *Leishmania* chromosomes show a high size polymorphism when comparisons are made between different isolates or between variants of the same strain. The genetic basis for this polymorphism is unknown; nevertheless, evidence suggests that mechanisms of DNA amplification/deletion and chromosomal rearrangement, e.g., translocation, are probably involved.[32] This feature of chromosomal size variability has been shown in other parasitic protozoa such as *Giardia lamblia*,[33] *Plasmodium falciparum*[34] and *Trypanosoma brucei*[35] suggesting an extensive genomic plasticity in these parasites. The detection of several genetic markers has allowed a more comprehensive evaluation of the chromosomal identity,[36] showing in some particular cases, physical linkage groups in the smaller chromosomes.[37]

Nuclear DNA

Analysis of the *Leishmania* genome using reassociation kinetics has shown three different kinetic components. The first component consists of repetitive sequences (>10^3 copies) constituting about 25% of the genome; a second component representing the moderately repetitive sequences (≈10-10^2 copies) comprising 13% of the genome; and the last component, the unique sequences constituting 60% of the genome. In comparison with other Kinetoplastida, the single copy DNA fraction in *Leishmania sp.* is three times higher than in *Trypanosoma cruzi*[38] and very similar to *Trypanosoma brucei*.[39]

The analysis of repetitive sequences has shown more heterogeneity within a single species than among different ones, despite copy number, function or genomic distribution. This, suggests that repetitive sequences have evolved in a concerted manner, and provides a specific target for the precise characterization and identification of distinct organisms. In Kinetoplastida, repetitive sequences have been isolated and their sensitivity and specificity in the identification of parasites have been widely proved (see chapter 6).[40-42] One of these repetitive sequences is the well-characterized 39 nt splice leader sequence (SLS) or miniexon, which is acquired by trans-splicing of the nuclear mRNAs.[43,44] Other repetitive sequences have been identified in small chromosome DNA bands in some *Leishmania* species (see chapter 6).

Kinetoplast DNA

Kinetoplast DNA (kDNA) represents the mitochondrial DNA (mtDNA) of the Kinetoplastida and corresponds to 10-20% of the total DNA.[45,46] Compared to the uniformity of mtDNA in animals and plants, kDNA is unusual in terms of structure. Intact kDNA contains two circular molecules, the homogeneous maxicircle (≈50 molecules of 20 kb) and the heterogeneous minicircle (0.8 kb in *Leishmania sp.*), which has many copies (≈10^4). Both molecules are concatenated into

a network. The maxicircle is the functional counterpart of the mitochondrial DNA in other eukaryotic cells; nevertheless, the gene expression of this molecule involves a complex process termed editing.[47,48] The minicircle has been associated with structural functions. Recently, a role in the editing of some maxicircle genes has been demonstrated.[47]

METHODS FOR THE CHARACTERIZATION OF *LEISHMANIA* PARASITES

From the epidemiological point of view, the characterization at the subgenera and species levels of *Leishmania* parasites is very important to establish the precise geographical distribution and mode of transmission of these human pathogens. This aspect is also important in terms of clinical diagnosis and evaluation of therapeutical schemes for the disease.

For many years, the unequivocal diagnosis of leishmaniasis depended on the direct observation of the parasite in a histological or smear sample. This procedure is insensitive and fails to identify the subgenera and species of parasite. Other biological, biochemical, and immunological parameters have been used to attempt identification at the species level. For example, properties such as the buoyant density of nuclear or kinetoplast DNA,[49] electrophoretic mobility of different isoenzymes,[50-53] distinct growth patterns in culture media, sandfly or hamster,[43,54] and ultrastructural size differences[55,56] may identify specific *Leishmania* species. However all these techniques require the culturing of ample numbers of parasites for analysis. For that reason, non-invasive immunological and molecular probes have been developed to allow rapid and precise characterization of the parasite from small samples.

Immunological Methods

The Program for Research and Training in Tropical Diseases (TDR) of the WHO has concentrated effort in the development of immunological tests for the detection of acute cases of visceral leishmaniasis.[57] This effort has produced Direct Agglutination Tests (DAT) and Enzyme-Linked Immunosorbent Assays (ELISA) for the fast detection of antibodies and parasite antigen in cases of visceral leishmaniasis.

Various investigators have developed monoclonal antibodies that allow the identification of the parasite at both subgenus and species levels.[58-60] In 1990, a call for submission of *Leishmania*-specific monoclonal antibodies was made by TDR. The screening of these reagents on the grounds of specificity, affinity and stability by groups of experts rendered the final selection of fifteen monoclonal antibodies. The hybridoma cell lines of these antibodies are deposited at the American Type Culture Collection (ATCC) for worldwide distribution, and TDR helps to provide them to centers involved in clinical trials and parasite characterization.

The intradermal delayed-type hypersensitivity skin test has been used as a support for diagnosis and in epidemiological studies as a

confirmation of exposure to the parasite. Since the original skin test—referred to as the Montenegro reaction—involves the use formalin-killed entire parasites, the more general term of leishmanin is currently used and includes purified *Leishmania* antigens, or whole parasites killed by formalin, heat or other means. TDR has made an effort for standardization and high quality control of the various antigens to be used in leishmanin skin test throughout the world.

Biochemical Methods

The use of electrophoresis in the study of enzyme polymorphisms is a valuable and frequently used tool for the identification of *Leishmania*.[61] Particular isoenzyme band patterns (zymodemes) are potentially useful in the classification of species. Important taxonomic information can be obtained by numerical analysis of the electrophoretic bands, which may vary according to the different alleles or genotypic frequencies of loci that are present in distinct parasite strain. Recently, Cupolillo et al[62] showed that only some enzymes produce diagnostic alleles. It was shown that 6-phospho-gluconate dehydrogenase was the most polymorphic enzyme, containing the majority of diagnostic alleles, and could be used to separate strain of the subgenus *Leishmania* from those of *Viannia*.

Molecular Probes

The most commonly used DNA in the identification of *Leishmania* parasites is the kDNA. Sequencing and comparison of minicircle classes among species generate information about the evolutionary divergence of particular regions, which could be used in the preparation of specific probes. Despite the heterogeneity of the minicircle population and the apparently high evolutionary rate, this molecule provides sequences with an appropriate level of variability that could be used efficiently in the identification of *Leishmania*. The degree of sensitivity using these DNA probes allows identification at the subgenus level and in particular cases enables interspecific discrimination (see chapter 6).

Repetitive sequences of nuclear DNA are probably the most convenient diagnostic targets on the parasite genome. Repetitive sequences are highly conserved but show sufficient divergence among related species to allow high diversity and specificity of the probes. Oligonucleotide primers from specific repetitive sequences can be used with the polymerase chain reaction (PCR), permitting discrimination among the major groups of *Leishmania*. Although, the characterization of the parasites using nuclear DNA probes has failed to show inter or intraspecific differences, the finding of highly polymorphic areas in the β-tubulin gene of *Leishmania* has proved sensitive at this level (see chapter 6). In addition, recent investigations on the distribution of specific DNA

sequences on chromosomes showed an association for *L. (V.) peruviana* of a north to south cline[63,64] and using these same techniques *L. (V.) braziliensis* and *L. (V.) peruviana* clustered separately.

Reference Strains and Strain Definitions

The necessity of reference strains became important as more workers used *Leishmania*. Participants of a WHO sponsored international meeting[65] held in 1980 proposed that reference strains should be chosen for each species. They also recommended that a standardized International Code Number (ICN) or strain numbering system should be adopted for *Leishmania* isolates. The concept of a reference strain is covered by the Zoological Nomenclature Code that requires that a specimen is chosen as the type of a newly described species. In most animals the specimen is a single dead individual stored in a museum collection. Initially *Leishmania* species were named because they produced unique pathologies, and since all were considered morphologically similar, no type specimens were chosen.

The International Code Number for *Leishmania* strains is composed of four parts. The first is the host code, the second is the country code, the third is the year of isolation and the fourth is the original strain number. The host and country codes can be found in WHO publications[66,67] and on the Base Dados Tropicais (http://www.bdt.org.br) Web pages of the International Leishmaniasis Network (ILN). In the case of a sandfly the host code is preceded by I, and for a mammal by M. Thus the International Code Number for a strain from a man infected in Costa Rica which was isolated in 1994 and referred to as strain H15, would be MHOM/CR/94/H15. The International Code number of the strain must be given in full, at least once, in any publication and the source of the strain should be mentioned.

Studies on laboratory-maintained isolates began to suggest, however, that these superficially similar parasites were not the same and in certain laboratories specific isolates from distinct geographical regions were compared by different methods. Although the methods varied, it became clear that if characters were defined and compared between laboratories they would have to relate to a single isolate. It was this idea that motivated the creation of reference strains. Cryopreservation reduced variations associated with continued serial passaging, although many older reference strains recommended by WHO[67] (Table 1.1) have been passaged in either culture or hamsters for many years.

Recently descriptions of new species have either selected a single strain as the type specimen or a series of strains. In the latter case the strains are paratypes. For *Leishmania* a strain rather than a single individual becomes the type material. One difficulty that we presently face is that some reference strains have been maintained in the laboratory for many years and may no longer have all the characteristics of the original population. This has led to a conservative approach in which

reference strains have not been designated as neotype material for the species that they represent. The reference strain of each species is one that has been extensively studied and characterized and that groups of experts or a specialized laboratory also consider representative of the species. It is only possible to know if an old reference strain is a reliable example of a species when it is compared to new isolates from the host and location from which the species was originally described. A reference strain may or may not be the holotype, neotype or a paratype of a species.

Table 1.1. International reference strains based on the list published by WHO[61] and recent descriptions of new species

Leishmania species	Location	Reference Strain
L.(L.)aethiopica	Old World	MHOM/ET/72/L100
*L.(L.)amazonensis***	New World	MHOM/BR/73/M2269
*L.(L.)arabica**	Old World	MPSA/SA/83/JISH220
L.(L.)archibaldi	Old World	not chosen
*L.(L.)aristedesi***	New World	MORY/PA/69/GML3
L.(L.)chagasi	New World	MHOM/BR/PP75
L.(L.)deanei	New World	MCOE/BR/74/M2674
L.(L.)donovani	Old World	MHOM/IN/80/DD8
*L.(L.)enriettii**	New World	MCAV/BR/45/L88
*L.(V.)forattinii**	New World	MDID/BR/77/Conchas
L.(L.)garnhami	New World	MHOM/VE/76/JAP78
L.(L.)gerbilli	Old World	MRHO/CN/60/GERBILLI
L.(L.)hertigi	New World	MCOE/PA/80/65/C8
*L.(L.)infantum***	Old World	MHOM/TN/80/IPT1
*L.(L.)killicki**	Old World	MHOM/TN/80/LEM904
L.(L.)major	Old World	MHOM/SU/73/5ASKH
L.(L.)mexicana	New World	MHOM/BZ/82/BEL21
L.(L.)pifanoi	New World	MHOM/VE/57/LL1
L.(L.)turanica	Old World	not chosen
L.(L.)tropica	Old World	MHOM/SU/74/K27
L.(L.)venezuelensis	New World	MHOM/VE/81/PMH17
L.(V.)braziliensis	New World	MHOM/BR/84/LTB300
*L.(V.)colombiensis**	New World	IHAR/CO/85/CL500
*L.(V.)equatorensis**	New World	MCHO/EC/82/Lspl
L.(V.)guyanensis	New World	MHOM/BR/75/M4147
*L.(V.)lainsoni**	New World	MHOM/BR/81/M6426
*L.(V.)naiffi**	New World	MDAS/BR/79/M5533
L.(V.)panamensis	New World	MHOM/PA/71/LS94
L.(V.)peruviana	New World	MHOM/PE/84/LC39[1]
*L.(V.)shawi**	New World	MCEB/BR/84/M8408

Type status of reference strain:* = holotype; ** = neotype.
[1] After figures given by Arana et al, 1990[58]

In any study it is recommended that reference strains are always included and that they be obtained from a reliable source. Inquires as to how best to obtain them can be obtained by consulting the International Leishmaniasis Network (e-mail address: Leish-L@bdt.org.br). Recently attention has been drawn[67] to the dangers of strains being mixed up, so constant confirmation of the correct identity of a reference strain is desirable, especially in the case of an exceptional result.

THE VECTOR

Leishmaniases are transmitted by sandflies (Order Diptera, Family Psychodidae, Subfamily Phlebotominae) of the genus *Phlebotomus* (Old World), *Lutzomyia* and *Psychodopygus* (*Ps*)(New World). Some entomologists consider the latter genus as a subgenus of the former.

The subfamily Phlebotominae is composed of minuscule, sand-colored biting flies that are widespread throughout the tropical and subtropical zones. There are far more sandfly species in the New World than the Old World and this is often quoted as the reason that there are more species of *Leishmania* in the New World. Table 1.2 lists the vectors of the different species. Details of the vectors and their relative importance can be found in several review articles.[11,69,70]

Sandflies usually repose during the day in burrows, tree hollows, caves or buildings. After sundown when the air temperature balances the underground temperature, they leave the shelters to remain active throughout the night. Air movements and changes in temperature may alter their nocturnal activity. This activity may be prolonged during daytime if temperature and light are appropriate.

The finding of an infection in a sandfly is one of the first phases in the incrimination of a vector, but other studies on the behavior of the fly and its vectorial capacity are necessary before it is considered as a vector.

The *Leishmania* parasites cause certain damage to the sandfly. During their travels through the gut of the sandfly, the procyclic promastigotes are transformed into metacyclic promastigotes in a process that involves structural modifications and enzymatic damage of the cardiac valve that normally prevents reflux from the gut to the pharynx.[71,72]

Genetic Variability of Sandflies

As with any other insect populations there is bound to be genetic variation among sandfly populations. There are differences in susceptibility to infection of different individuals of the same population and it is possible that there are also variations in the degree of host preference within a species.[73,74] Such genetically controlled characters would greatly influence the vectorial importance of a sandfly population in a particular geographical area.

It has been shown that morphologically similar populations of *Lutzomyia (Lu.) longipalpis* fail to interbreed and that they have different pheromones.[75-77] These results suggest that *Lu.longipalpis* is a com-

plex of sibling species. Studies on sandflies identified as ***Lu.whitmani*** from different regions revealed small morphological differences and variations in their response to a DNA probe suggest that ***Lu.whitmani*** is a complex of morphologically similar species.[78] The genetic homogeneity of other sandfly vectors that are morphologically similar and have extensive geographical distributions has not been investigated.

***Table 1.2. Vectors of Old and New World* Leishmania**

***Leishmania* Species**	**Vector–Sandfly species**
L.(L.) aethiopica	*P. longipes, P. pedifer*
L.(L.) amazonensis	*Lu. flaviscutellata, Lu. olmeca nociva*, Lu. reducta**
L.(L.) arabica	unknown
L.(L.) archibaldi	*P. orientalis, P. martini*
L.(L.) aristedesi	suspected–*Lu. olmeca bicolor*
L.(L.) chagasi	*Lu. longipalpis, Lu. evansi*
L.(L.) deanei	unknown
L.(L.) donovani	*P. argentipes*
L.(L.) enriettii	suspected *Lu. monticola*
L.(V.) forattinii	suspected *Lu. yuilli and Ps. ayrozai*
L.(L.) garnhami	suspected–*Lu. youngi*
L.(L.) gerbilli	unknown
L.(L.) hertigi	unknown
L.(L.) infantum	*P. ariasi; P. perniciosus*
L.(L.) killicki	unknown
L.(L.) major	*P. papatasi; P. duboscqi; P. salehi*
L.(L.) mexicana	*Lu. olmeca olmeca*
L.(L.) pifanoi	unknown
L.(L.) turanica	*P. andrejevi, P. papatasi*
L.(L.) tropica	*P. sergenti*
L.(L.) venezuelensis	suspected *Lu. olmeca bicolor*
L.(V.) braziliensis	*Ps. carrerai, Ps. complexus, Ps. llanosmartini. Ps.wellcomei, Ps. yucumensis, Lu. intermedia, Lu. spinicrassa, Lu. whitmani.*
L.(V.) colombiensis	*Lu. hartmanni*, Lu. gomezi*, Ps. panamensis**
L.(V.) equatorensis	unknown
L.(V.) guyanensis	*Lu. umbratilis, Lu. anduzei, Lu. whitmani,*
L.(V.) lainsoni	*Lu. ubiquitalis*
L.(V.) naiffi	*Ps. ayrozai, Ps. paraensis, Ps. squimiventris*
L.(V.) panamensis	*Lu. trapidoi, Lu. ylephilator, Lu. gomezi, Ps. panamensis*
L.(V.) peruviana	*Lu. peruensis,* suspected *Lu. verrucarum*
L.(V.) shawi	*Lu. whitmani*

Lu.= *Lutzomyia*; P.= *Phlebotomus*; Ps.= *Psychodopygus*.
* found naturally infected but importance as vector undetermined.

Identification Methods for Sandflies

Morphology of the males and females is the main identification method for sandflies but the morphology of the male and female genitalia are particularly important. In some closely related species, however, the male or female genitalia may be the same. A definitive identification then depends on the morphology of the genitalia of the other sex. In the Amazonian region of Brazil the females of two vectors of *L.(V.)braziliensis*, *Ps.wellcomei* and *Ps.complexus* are morphologically indistinguishable. This problem was resolved by the development of a species specific DNA probe for *Ps.wellcomei*.[79] A species specific probe has also been made for *P. papatasi* that fail to react with the morphologically similar species, *Ps. duboscqu*.[80]

Vector Control

The control of the vector by insecticide spraying is one of the recommended methods of controlling outbreaks of leishmaniasis in human populations when transmission is peridomestic.[61] In the past malaria vector control programs have been attributed as also being responsible for the control of leishmaniasis. The reappearance of visceral leishmaniasis in India is thought be associated with an increase in the numbers of *P.argentipes* that occurred when the malaria control program stopped using insecticides. The same is also thought to be true for certain regions of Brazil.

Controlling of sylvanic species of sandflies is not possible due to the enormous size of the areas involved. Modifications of the environment, such as the cutting of the native forests, may reduce or even eliminate certain vector species, but some can adapt to even man made forests.[81]

The use of insecticides in agriculture may also have some influence on populations of sandflies. Recent criticism of their indiscriminate use and effect on the environment has resulted in them being used more critically. This could be another factor effecting the increase in peridomestic sandfly populations that has occurred in the 1990s, especially in the New World.

Avoidance of contact with the vector is the only method available in reducing the man/sandfly contact in many situations. Some investigators say repellents are useful while others say they are not. Only fine netting is useful in protecting against sandflies and the mesh usually used for mosquitoes bed nets does not impede sandflies. Experimental trials are in progress on the use of insecticide treated netting which may function because of the repellent effect of the insecticide.

THE LEISHMANIASES

Leishmaniasis provides an excellent model to understand the diverse regulatory mechanisms that mediate host resistance and susceptibility to disease, and has implications for other infectious diseases. This is due both to the existence of experimental models of leishmaniasis

in different inbred strains of mice and to the diversity of the immune response to *Leishmania* in humans.

The leishmaniases occur in three major forms: visceral, cutaneous and mucocutaneous.

Visceral Leishmaniasis and Post-Kala-Azar Dermal Leishmaniasis

In visceral leishmaniasis (VL), the parasite invades internal organs (spleen, liver, bone marrow, etc.) and the consequences are usually fatal if untreated. This disease, also known by the Indian name of kala-azar, is endemic in several parts of Africa, India and Latin America, and occurs sporadically in China, the Mediterranean Basin and South West Asia. The parasites involved in this serious illness are *L. (L.) donovani* and *L. (L.) chagasi* in the Old and New Worlds respectively. Histologically, the parasites may be found in macrophages of the spleen, liver, lymph nodes and even the skin (post-kala-azar dermal leishmaniasis, PKDL). Immunologically, VL patients show a selective anergy to *Leishmania* antigens, first characterized by a negative leishmanin test, which fail to convert even after cure.

PKDL is usually manifested as a complication of VL, and it is considered the major source of VL transmission in the Indian subcontinent.[82] Indian PKDL develops 1-5 years after the apparent clinical cure of the visceral disease. These lesions persist for many years and seldom ulcerate. The lesions are clinically similar to DCL but the histology is more complex with parasite-filled macrophages.[82] In contrast, the infrequent African PKDL develops during treatment before the resolution of VL, and it usually associated with good prognosis.[82]

Cutaneous and Mucocutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is the most widespread form of the disease, found in Africa, the Indian subcontinent, Latin America, South-West Asia, and part of the Mediterranean Basin. CL is characterized by several different clinical, histopathological and immunological features. Of these, cell-mediated immunity is generally believed to be of dominant influence in determining the outcome of the disease. The disease spectrum of CL includes immune-responder individuals with the localized cutaneous form (LCL), characterized by an adequate cell-mediated immune (CMI) response and well-defined skin lesions, and non-responder individuals with disseminated or diffuse cutaneous leishmaniasis (DCL), which occurs infrequently and is characterized by a selective anergy in CMI, resulting in extensive involvement of the skin, naso-bucopharyngeal mucous tissue and some lymph nodes.[83-85]

In the New World, the cutaneous disease is termed American cutaneous leishmaniasis (ACL), and is also manifested as a spectrum of clinical, histopathological and immunological manifestations.[86,87]

A third entity, mucocutaneous leishmaniasis (MCL), is characterized by exacerbated CMI and destructive lesions of the oral and

nasopharyngeal cavities that may appear long after the healing of the primary skin lesions.[87-89] This form is more common in the Americas but it has been observed in the Old World.[82]

Histologically, LCL granulomas are composed of a prominent infiltration of lymphocytes, variable numbers of epithelioid cells, and few parasites. Ulcerated lesions show variable degrees of epidermal cell hyperplasia, which depends on the time of evolution and type of lesions.[90] DCL lesions contain many undifferentiated macrophages laden with parasites, with few lymphocytes and plasma cells.[90] The MCL granuloma is a mixture of lymphocytes and macrophages, with very few parasites and moderate epidermal changes.[91] In the Americas, parasite species also may be associated with the clinical spectrum, since parasites from the *L. (L.) mexicana* and *L. (V.) braziliensis* complexes have exclusively been found in DCL and MCL lesions, respectively.[92]

Experimental Cutaneous Leishmaniasis

In mice, depending on the animal strain, *Leishmania* strain and the number of inoculated parasites, it has been possible to reproduce the distinct clinical forms observed in humans (see chapter 7). Thus, syngeneic strains C57BL/6, CBA, C3H/He and AKR are resistant to *L. (L.) major* and *L. (L.) mexicana* and develop skin lesions that spontaneously resolve similar to LCL lesions.[93,94] In contrast, BALB/c, DBA/2 and A/Jax mice are susceptible and develop chronic and progressive lesions similar to DCL.[94,95] The intermediate forms of the disease can be reproduced in CBA, NMRI and B.10D2 strains.[94,96]

The established experimental lesion in BALB/c involves an active inflammatory response with participation of the epidermis and dermis.[97,98] The murine model of cutaneous leishmaniasis is extremely important for the analysis of the cellular response leading to the resolution of lesions induced by the parasite.

Acknowledgments

This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Consejo de Desarrollo Científico y Humanístico-Universidad Central de Venezuela grants 03.10.2831.94 and PC03.004.91 (AM-L) and 09-35-2379-93 (FJT), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT) grants S1-2469 and RPIV-110034 (AM-L), CONICIT-Banco Interamericano de Desarrollo (BID) grant BTS-60 (FJT), and Fundación Polar.

References

1. Leishman WB. On the possibility of the occurrance of trypanosomiasis in India. Br Med J 1903; 1:1252-4.
2. Kassai T, Burt MDB. A plea for consistency. Parasitol Today 1994; 10:127-8.

3. Modabber F. Leishmaniasis. In: Tropical Disease Research. Progress 1991-92. Eleventh Programme report of the UNDP/world Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). Geneva: World Health Organization, 1993:77-87.
4. Molyneux DH, Killick-Kendrick R. Morphology, ultrastructure and life cycles. In: Peters W, Killick-Kendrick R, eds. The Leishmaniases in Biology and Medicine. London: Academic Press, 1987:121-176.
5. Janovy J, Lee KW, Brumbaugh JA. The differentiation of *Herpetomonas megaseliae*: ultrastructural observations. J Protozool 1974; 21:53-9.
6. Da Silva R, Sacks DL. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect Immun 1987; 55:2802-6.
7. Mauël, J. Macrophage-parasite interactions in *Leishmania* infections. J Leuk Biol 1990; 47:187-192.
8. Blank C, Fuchs H, Rappersberger K et al. Parasitism of epidermal Langerhans cells in experimental cutaneous leishmaniasis. J Inf Dis 1993; 167:418-25.
9. van Wilsem EJG, Brevé J, Kleijmeer M et al. Antigen-bearing Langerhans cells in skin draining lymph nodes: Phenotype and kinetics of migration. J Invest Dermatol 1994; 103:217-20.
10. Moll H, Flohé C, Röllinghoff M. Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. Eur J Immunol 1995; 25:693-9.
11. Lainson R, Shaw JJ. Evolution, classification and geographical distribution. In: Peters W, Killick-Kendrick R, eds. The Leishmaniases in Biology and Medicine. London: Academic Press, 1987:1-120.
12. Pifano F. Algunos aspectos en la patología comparada geográfica de la leishmaniasis tegumentaria en el trópico americano. Gaceta Médica de Caracas 1960; 28:1-3.
13. Medina R, Romero J. Estudio sobre la leishmaniasis tegumentaria en Venezuela. Dermatol Ven 1957; 1:30-86.
14. Lainson R, Shaw JJ. Leishmaniasis in the New World: taxonomic problems. Br Med Bull 1972; 28:44-8.
15. Lainson R, Shaw JJ. Leishmaniasis in the New World, with particular reference to Brazil. Bull PAHO 1973; 7:1-19.
16. Lainson R, Shaw JJ. The epidemiology and ecology of Leishmaniasis in Latin America. Nature 1978; 273:595-600.
17. Lainson R, Shaw JJ. The role of animals in the epidemiology of South American leishmaniasis. In: Lumsden WHR, Evans DA, eds. Biology of the Kinetoplastida. London: Academic Press, 1979:1-116.
18. Shaw JJ. Taxonomy of the genus *Leishmania*: traditionalist's view and modern concepts. In: Biochemical Characterization of *Leishmania*. Proceedings of a Workshop held at the Pan American Health Organization, 9-11 December 1980. Geneva: UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1982:9-24.
19. Fernandes O, Murthy VK, Kurath U et al. Mini-exon gene variation in

human pathogenic *Leishmania* species. Mol Biochem Parasitol 1994; 66:261-71.
20. Shaw, JJ. Taxonomy of the Genus *Leishmania*: Present and Future Trends and Their Implications. Mem Inst Oswaldo Cruz 1994; 89:471-78.
21. Rioux J-A, Lanotte G, Serres E et al. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comp 1990; 65:111-25.
22. Cupolillo E, Grimaldi Jr G, Momen H. A general classification of the New World *Leishmania* using numerical zymotaxonomy. Am J Trop Med Hyg 1994; 50:296-311.
23. Cupolillo E, Grimaldi Jr G, Momem H et al. Intergenic region typing (IRT): A rapid molecular approach to the characterization and evolution of *Leishmania*. Mol Biochem Parasitol 1995; 73:145-55.
24. Beverley SM. Gene amplification in *Leishmania*. Ann Rev Microbiol 1991; 45:417-44.
25. Liu J, Gajendran N, Muthui D et al. Chromosome rearrangement in *Leishmania mexicana* M379. Mol Biochem Parasitol 1991; 46:53-60.
26. Wang AL, Wang CC. Viruses of parasitic protozoa. Parasitol Today 1991; 7:76-80.
27. Cruz A, Beverley SM. Gene replacement in parasitic protozoa. Nature 1990; 348:171-3.
28. Coburn CM, Otteman KM, McNeely T et al. Stable DNA transfection of a wide range of trypanosomatids. Mol Biochem Parasitol 1991; 46:169-80.
29. Wesley RD, Simpson L. Studies of kinetoplast DNA. III. Kinetic complexity of kinetoplast and nuclear DNA from *Leishmania tarentolae*. Biochim Biophys Acta 1973; 319:267-80.
30. Leon W, Fouts DL, Manning J. Sequences arrangement of the pathogenic haemoflagellate *Leishmania donovani*. Nucl Acids Res 1978; 5:491-505.
31. Schwartz DC, Cantor CR. Separation of yeast chromosome-sized DNAs by pulse field gradient gel electrophoresis. Cell 1984; 37:67-75.
32. Pagès M, Bastien P, Veas F et al. Chromosome size and number polymorphisms in *Leishmania infantum* suggest amplification/deletion and possible genetic exchange. Mol Biochem Parasitol 1989; 36:67-75.
33. Le Blancq SM. Chromosome rearrangements in *Giardia lamblia*. Parasitol Today 1994; 10:177-9.
34. Ravetch JV. Chromosomal polymorphisms and gene expression in *Plasmodium falciparum*. Exp Parasitol 1989; 68:121-5.
35. Van der Ploeg L. Antigenic variation in African trypanosomes: genetic recombination and transcriptional control VSG genes. In: Hames BD, Glover DM, eds. Gene Rearrangement. Oxford: IRL Press, 1990:51-97.
36. Giannini SHM, Schittini M, Keithly JS et al. Kariotype analysis of *Leishmania* species and its use in classification and clinical diagnosis. Science 1986; 232:762-5.
37. Ravel CD, Macari F, Bastien P et al. Conservation among Old World *Leishmania* species of six physical linkage groups defined in *Leishmania*

infantum small chromosomes. Mol Biochem Parasitol 1995; 69:1-8.
38. Lanar D, Levy LS, Manning JE. Complexity and content of the DNA and RNA in *Trypanosoma cruzi*. Mol Biochem Parasitol 1981; 3:327-41.
39. Borst P, Van der Ploeg M, Van Hoek JFM et al. On the DNA content and ploidy of trypanosomes. Mol Biochem Parasitol 1982; 6:13-23.
40. Hide G, Cattand P, Le Ray D et al. The identification of *Trypanosoma brucei* subspecies using repetitive DNA sequences. Mol Biochem Parasitol 1990; 39:213-26.
41. Wincker P, Raizes G, Goldenberg S. Characterisation of *Trypanosoma cruzi* specific nuclear repeated sequence. Mol Biochem Parasitol 1990; 41:147-52.
42. Eresh S, de Bruijn MHL, Mendoza-León A et al. *Leishmania (Viannia) lainsoni* occupies a unique niche within the subgenus *Viannia*. Trans R Soc Trop Med Hyg 1995; 89:231-6.
43. Vanhamme L, Pay E. Controls of gene expression in Trypanosomes. Microbiol Rev 1995; 59:223-40.
44. Graham SV. Mechanism of stage-regulated gene expression in Kinetoplastida. Parasitol Today 1995; 11:217-23.
45. Englund PT, Ferguson M, Guilbride DL et al. The replication of kinetoplast DNA. In: Boothroyd JC, Komuniecki R, eds. Molecular Approaches to Parasitology, MBL Lectures in Biology. New York: Wiley-Liss, 1995:147-61.
46. Simpson L. The mitochondrial genome of Kinetoplastida: transcription, replication and evolution. Ann Rev Microbiol 1987; 41:363-82.
47. Simpson L, Shaw JJ. RNA editing and the mitochondrial cryptogenes of kinetoplastid protozoa. Cell 1989; 57:355-66.
48. Benne R. RNA editing in trypanosomes: is there a message? Trends Genet 1990; 6:177-81.
49. Chance ML, Peters W, Shchory L. Biochemical taxonomy of *Leishmania*. I. Observation on DNA. Ann Trop Med Parasit 1974; 68:307-16.
50. Gardener PJ, Chance ML, Peters W. Biochemical taxonomy of *Leishmania*. II. Electrophoretic variation of malate dehydrogenase. Am Trop Med Parasitol 1974; 68:317-26.
51. Kilgour V, Gardener PJ, Godfrey DG, Peters W. Demonstration of electrophoretic variation of two amino transferase in *Leishmania*. Am Trop Med Parasitol 1974; 68:245-6.
52. Martin E, Simon MW, Schaefer FW, Muhhada AJJ. Enzymes of carbohydrate metabolisms in four human species of *Leishmania*: A comparative survey. Protozool 1978; 23:600-7.
53. Miles MA, Povoa MM, De Souza AA, Lainson R, Shaw JJ. Some methods for the enzyme characterization of Latin American *Leishmania* with particular reference to *Leishmania mexicana amazonensis* and subspecies of *Leishmania hertigi*. Trans R Soc Trop Med Hyg 1979; 74:243-52.
54. Zucherman A, Lainson R. *Leishmania*. In: Kreier JP, ed. Parasitic Protozoa, Vol. 1. New York: Academic Press, 1977:57-61.
55. Shaw JJ, Lainson R. Leishmaniasis in Brazil. XI Observations on the

morphology of *Leishmania* of the *braziliensis* and *mexicana* complexes. J Trop Med Hyg 1976; 79:9-13.
56. Gardener PJ, Schory L, Chance ML. Species differentiation in the genus *Leishmania* by morphometric studies with the electron microscope. Ann Trop Med Parasit 1977; 71:147-55.
57. Chowdhury S, Haque F, Al.Masum A, El Harith A, Karim E. Positive response to sodium antimony gluconate administration in visceral leishmaniasis seropositive patients. Am J Trop Med Hyg 1991; 44:390-3.
58. McMahon-Pratt D, David JR. Monoclonal antibodies that distinguish between New World species of *Leishmania*. Nature 1981; 291:581-83.
59. Anthony RL, Williams KM, Sacci JB, Rubin DC. Subcellular and taxonomic specificity of monoclonal antibodies to New World *Leishmania*. Am J Trop Med Hyg 1985; 34:1085-94.
60. Grimaldi Gjr, David JR, MacMahon-Pratt D. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. Am J Trop Med Hyg 1987; 36:270-87.
61. World Health Organization. Control of Leishmaniases. Technical Report Series Nº 793. Geneva: World Health Organization, 1990.
62. Cupolillo E, Grimaldi G, Momen H. Discrimination of *Leishmania* isolates using a limited set of enzymatic loci. Ann Trop Med Parasitol 1995; 89:17-23.
63. Dujardin JC, Bañuls A-L, Llanos-Cuentas A et al. Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Trop 1995; 59:293-307.
64. Dujardin JC, Dujardin JP, Tibayrenc M et al. Karyotype plasticity in Neotropical *Leishmania*: an index for measuring genomic distance among *L. (V.) peruviana* and *L. (V.) braziliensis* populations. Parasitology 1995; 110:21-30.
65. World Health Organization. Recommendations. In: Chance ML, Walton BC, eds. Biochemical Characterization of *Leishmania*. UNDP/WORLD BANK/WHO 1982; 263-76.
66. World Health Organization. The Leishmaniases. Report of a WHO Expert Committee. Wld Hlth Org Techn Rep Ser 1984; 701:1-140.
67. Shaw JJ, Camargo EP. Are Trees Real? Parasitol Today 1995; 11:347.
68. Arana A, Evans DA, Zolessi A et al. J. Biochemical characterization of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *peruviana* by isoenzyme electrophoresis. Trans R Soc Trop Med Hyg 1990; 84:526-9.
69. Grimaldi GJ, Tesh RB, McMahon-Pratt D. A review if the geographic distribution and epidemiology of leishmaniasis in the New World. Am J Trop Med Hyg 1989; 41:687-725.
70. Young DG, Duncan MA . Guide to the identification and geographic distribution of *Lutzomyia* sandflies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Memoires of the American Entomological Institute no. 54. Gainsville: Associated Publishers, 1994:1-881.
71. Sacks DL, Pimenta PFP, McConville MJ et al. Stage-specific binding of *Leishmania donovani* to the sandfly vector midgut is regulated by confor-

mational changes in the abundant surface lipophosphoglycan. J Exp Med 1995; 181:685-97.
72. Schlein Y, Jacobson RL, Messer G. *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. Proc Natl Acad Sci USA 1992; 89:9944-8.
73. Lanzaro GC, Warburg A. Genetic variability in phlebotomine sandflies: Possible implications for leishmaniasis epidemiology. Parasitol Today 1995; 11:151-4.
74. Shaw JJ. The behaviour of *Endotrypanum schaudinni* (Kinetoplastidae: Trypanosomatidae) in three species of laboratory-bred neotropical sandflies (Diptera:Psychodidae) and its influence on the classification of the genus *Leishmania*. In: Canning EU, ed. Parasitological Topics, A Presentation Volume to P.C.C. Garnham F.R.S. on the occasion of his 80th Birthday 1981. Society of Protozoologists, Special Publication No 1. Lawrence: Allen Press, 1981:232-41.
75. Ward RD, Ribeiro AL, Ready PD, Murtagh A. Reproductive isolation between different forms of *Lutzomyia longipalpis* (Lutz & Neiva), (Diptera:Psychodidae), the vector of *Leishmania donovani chagasi* Cunha & Chagas and its significance to kala-azar distribution in South America. Mem Inst Oswaldo Cruz 1983; 78:269-80.
76. Ward RD, Phillips A, Burnet A, Marcondes CR. The *Lutzomyia longipalpis* complex: reproduction and distribution. In: Service MW, ed. Biosystematics of haematophagus insects. The Sytematics Association Special Volume No. 37. Oxford: Clarendon Press, 1988:257-69.
77. Ward RD, Hamilton JGC, Dougherty M, Falcão AL. Pheromones in Old and New World sandflies (Diptera:Pyschodidae). Ann Trop Med Parasit 1991; 85:667-8.
78. Rangel EF, Lainson R, Souza AA, Ready P, Azevedo ACR. Geographical variation in populations of *Lutzomyia* (*Nyssomyia*) *whitmani* (Antunes & Coutinho, 1939) *sensu lato* (Diptera: Psychodidae: Phlebotomine) in Brazil. Mem Inst Oswaldo Cruz 1996; 91:43-50.
79. Ready PD, Lainson R, Shaw JJ, Souza A. DNA probes for distinguishing *Psychodopygus wellcomei* from *Psychodopygus complexus* (Diptera: Psychodidae). Mem Inst Oswaldo Cruz 1991; 86:41-9.
80. Ready PD, Smith DF, Killick-Kendrick R. DNA hybridization on squash-blotted sandflies to identify both *Phlebotomus papatasi* and infecting *Leishmania major*. Med Vet Ent 1988; 2:109-16.
81. Ready PD, Lainson R, Shaw JJ: Leishmaniasis in Brazil: XX. Prevalence of "enzootic rodent leishmaniasis" (*Leishmania mexicana amazonensis*), an apparent absence of "pian bois" (*Le. braziliensis guyanensis*), in plantations of introduced tree species and in other non-climax forests of eastern Amazonia. Trans R Soc Trop Med Hyg 1983; 77:775-85.
82. Rees PH, Kager PA. Visceral leishmaniasis and post-kala-azar dermal leishmaniasis. In: Peters W, Killick-Kendrick R, eds. The Leishmaniases in Biology and Medicine. London: Academic Press, 1987:583-615.
83. Griffiths WAD. Old World cutaneous leishmaniasis. In: Peters W, Killick-

Epidermal Langerhans cells and dendritic epidermal T cells in murine cutaneous leishmaniasis. Immunocytochemical study. Acta Micros 1993; 2:180-7.

99. Lainson R, Braga RR, De Souza AAA et al. *Leishmania (Viannia) shawi sp. n.*, a parasite of monkeys, sloths and nocyonids in Amazonian Brazil. Ann Parasitol Hum Comp 1989; 64:200-207.
100. Laison R, Shaw JJ. *Leishmania (Viannia) naiffi sp. n.*, a parasite of the armadillo, *Dasypus novemcintus* in Amazonian Brazil. Ann Parasitol Hum Comp 1989; 64:3-9.
101. Kreutzer RD, Corredor A, Grimaldi G Jr et al. Characyerization of Leishmania colombiense sp. n. (Kinetoplastida: Trypanosomatidae), a new parasite infecting humans, animals and phlebotomine sandflies in Colombia and Panama. Am J Trop Med Hyg 1991; 44:662-75.
102. Grimaldi G Jr, Hashiguchi Y, Kreutzer RD et al. Description of Leishmania equator ensis sp. n. (Kinetoplastida: Trypanosomatidae), a new parasite infecting arboreal mammals in Ecuador. Mem Inst Oswaldo Cruz 1992; 87:221-8.
103. Mendoza-Leon A, Gavercroft D, Barker DC. The RFLP analysis of the β-tubulin gene region in New World *Leishmania*. Parasitology 1995; 111:1-9.