Tropical Medicine and International Health

VOLUME 7 NO 8 PP 641–651 AUGUST 2002

Review: **Molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification**

Henk D. F. H. Schallig and Linda Oskam

Koninklijk Instituut voor de Tropen (KIT), Biomedical Research, Amsterdam, the Netherlands

Summary Molecular biology is increasingly relevant to the diagnosis and control of infectious diseases. Information on DNA sequences has been extensively exploited for the development of polymerase chain reaction-based assays for the diagnosis of leishmaniasis and the identification of parasite species. It has also led to the use of cloned antigen for serodiagnosis. It is expected that the sequencing of the *Leishmania major* genome and the genomes of other *Leishmania* species will enable important progress in further improving diagnosis and control. The ability to use genome data to clone and sequence genes, which, when expressed, provide antigens for vaccine development, will increase the possibilities for rational vaccine development. Moreover, DNA on its own will provide the basis for the development of DNA vaccines that may overcome some of the problems encountered with protein-based vaccines. One of the greatest threats to parasite control is the development of drug resistance in parasites. Knowing the molecular basis of drug resistance and the ability to monitor its development with sensitive and specific DNA-based assays for 'resistance alleles' may aid maintaining the effectiveness of available anti-*Leishmania* drugs. Finally, techniques such as microarrays and nucleic acid sequence-based amplification will eventually allow rapid screening for specific parasite genotypes and assist in diagnostic and epidemiological studies.

keywords *Leishmania*, cutaneous, visceral, leishmaniasis, molecular biology, diagnosis, control

correspondence Dr. Henk Schallig, KIT Biomedical Research, Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands. Fax: +31 20 697 1841; E-mail: h.schallig@kit.nl

Introduction

The World Health Organization (WHO) considers leishmaniasis to be one of the most important parasitic diseases with approximately 350 million people at risk of contracting the disease. Leishmaniasis has a worldwide distribution, it is endemic in at least 88 countries, and the disease occurs on all continents except Antarctica and Australia where no suitable vectors are present. There are three major clinical forms of the disease: (1) Visceral leishmaniasis (VL), affecting an estimated 500 000 people each year, is a deadly disease if left untreated. The parasites colonize the internal organs, in particular the spleen, liver, bone marrow and lymph nodes; (2) cutaneous and (3) muco-cutaneous leishmaniasis (CL and MCL, respectively) affecting approximately 1.5 million people per year. In the case of CL, the parasite is confined to the skin where it causes one or more slow-healing sores. In the case of MCL, the infection spreads to the mucosal membranes, especially those of mouth and nose, where it may cause extensive damage. More than 20 recognized *Leishmania* species are responsible for these diseases, each species having distinct epidemiological patterns.

Research in *Leishmania* has gained momentum in the last decade because of the application of molecular biology and is accelerated by the *Leishmania* genome project. This paper reviews some of the latest developments in the application of molecular tools in the diagnosis and control of leishmaniasis and parasite identification.

Diagnosis

The early diagnosis of leishmaniasis is important in order to avoid severe damage or even death of the patient. The routine diagnosis of leishmaniasis relies on either the microscopical demonstration of *Leishmania* amastigotes in aspirates from lymphoid tissue or liver, in slit skin smears or in peripheral blood or culturing. However, the retrieval

of samples is uncomfortable for the patient and the isolation of parasites by culturing is time-consuming, difficult and expensive. Because of these limitations, a number of indirect immunological methods, such as enzyme-linked immunosorbent assay (ELISA), dipsticks and direct agglutination test (DAT), have been developed. Despite the large number of serological tests that are available, there is still no gold standard diagnostic test. This is in part because of the fact that none of the tests is 100% sensitive and specific. Moreover, the spread of *Leishmania*/HIV coinfection complicates the use of the serological techniques as a result of low or lack of antibody responses of these patients (WHO 2000).

Identification of parasite antigens for serodiagnosis

Western blotting techniques have been extensively used to identify antigens for the serodiagnosis of (visceral) leishmaniasis. The most promising candidate so far is a 39 amino-acid repeat from a kinesin-like protein that is predominant in *Leishmania chagasi* tissue amastigotes (Burns *et al*. 1993). The recombinant protein rK39 was initially used in the ELISA, after which it was used for the development of a rapid immunochromatographic dipstick test (Jelinek *et al*. 1999). Both the ELISA and the dipstick test were tested for the diagnosis of visceral leishmaniasis. Initial reports were very promising: rK39-based tests were very sensitive and specific for visceral leishmaniasis, could be used in HIV-positive patients, and antibody levels against rK39 declined rapidly after successful treatment (Burns *et al*. 1993; Qu *et al*. 1994; Houghton *et al*. 1998). However, more recent reports show that the dipstick test lacks sensitivity (Jelinek *et al*. 1999; Zijlstra *et al*. 2001) and specificity (Veeken 2001). Currently, there are no other widely available diagnostic tests that are based on defined, cloned *Leishmania* antigen(s). The DAT remains the serological test of choice, in particular, in many developing countries (Schallig *et al*. 2001).

Diagnosis by polymerase chain reaction (PCR)

Over the years, a number of different PCR assays has been developed for the detection of *Leishmania* DNA in a variety of clinical samples such as skin biopsies and smears, bone marrow and lymph node aspirates and peripheral blood. Several target sequences have been used for the PCR. Maximum sensitivity can be achieved by using multicopy sequences as the PCR target (Lachaud *et al*. 2002). Examples of such targets are ribosomal RNA genes, kinetoplast DNA, mini-exon-derived RNA genes and genomic repeats (Osman 1998). The specificity of the PCR can be adapted to specific needs by targeting conserved or variable regions. In this way it is possible to characterize the parasite to the level of the genus complex, species or even the individual isolate.

For diagnosis of VL, bone marrow and lymph node aspirates as well as blood samples have been evaluated. Bone marrow aspirates from parasitologically confirmed VL patients were always PCR positive in several studies (Mathis & Deplazes 1995; Andresen *et al*. 1997; Osman *et al*. 1997a). In another study, comparison of PCR on bone marrow aspirates with microscopic examination and culture for diagnosis of VL in immune-compromised patients showed that PCR exhibited a higher sensitivity (82%) than microscopy (55%) and culture (55%) (Piarroux *et al*. 1994). In human lymph node aspirates *Leishmania* DNA was detected by PCR in all six samples in one study (Andresen *et al*. 1997) and in 33/38 samples in another (Osman *et al*. 1997a). In general, PCR is a more sensitive method for the detection of *Leishmania* in lymph node and especially bone marrow aspirates of VL patients than microscopy and is especially useful for the confirmation of cases of suspected VL.

Because lymph node, bone marrow and splenic aspiration is painful and can even be dangerous for the patient, peripheral blood, which is easy to obtain, may be used for the initial PCR screening of people suspected of having VL. The sensitivity of PCR for the detection of *Leishmania* DNA in blood samples ranges from around 70% (Adhya *et al*. 1995; Osman *et al*. 1997a) to 90% (Nuzum *et al*. 1995) and higher (Andresen *et al*. 1997; Salotra *et al*. 2001). It must be emphasized that if PCR on blood is negative, a PCR on lymph node and/or bone marrow material should be performed, because PCR on these materials is more often positive (Osman *et al*. 1997a).

PCR may also be useful for the confirmation of the diagnosis in HIV/*Leishmania* coinfected patients. Pizzuto *et al*. (2001) showed that all 76 HIV/*Leishmania* coinfected patients were parasitaemic by PCR on peripheral blood before therapy. In another study, 15 of 20 (75%) patients were PCR positive (Campino *et al*. 2000).

Patients with cutaneous or muco-cutaneous leishmaniasis (CL or MCL) often have low or no *Leishmania* antibodies, because of the localized character of the disease, and thus serological tests are mostly negative. Therefore, PCR is an important tool for the diagnosis of CL and MCL. Six of 10 skin biopsies from patients with CL were positive in PCR (Mathis & Deplazes 1995) and Andresen *et al*. (1996) found that 24 of 28 (86%) samples taken from CL patients were PCR positive, whereas microscopy detected parasites in only 55% of the samples that for reasons of epidemiology, clinical aspect, LST-status and pathology were considered to be CL. Later studies essentially confirmed these early findings with PCR

being positive in more than 90% of CL cases (Aviles *et al.* 1999; Pirmez *et al.* 1999). In MCL, PCR was capable of detecting parasites in 17 of 24 (71%) patients, whereas diagnosis by conventional techniques could only confirm the disease in four of 24 (17%) patients (Pirmez *et al.* 1999).

Also in post-kala-azar dermal leishmaniasis (PKDL) PCR has proved its value: when using slit skin smears from PKDL patients, 19 of 23 (83%) samples were PCR positive compared with only seven of 23 (30%) positive samples in microscopy (Osman *et al.* 1998a). In another study, 45 of 48 PKDL patients were PCR positive (Salotra *et al.* 2001).

Finally, PCR detection of parasite DNA in either lymph node aspirates or peripheral blood can be used as a prognostic marker for the development of relapse or PKDL after apparently successful treatment. Parasite DNA could still be detected by PCR in 80 and 40%, respectively, of the lymph node aspirates that were obtained from Sudanese VL patients either immediately after treatment (Osman *et al.* 1998) or at least 3 months after treatment (Osman *et al.* 1997b). While patients who were PCR negative remained free of signs and symptoms and were apparently cured, 36% of the patients with a positive PCR after treatment developed PKDL and 23% showed recurrence of VL symptoms with microscopic reappearance of parasites in the aspirates (Osman *et al.* 1998). In VL patients that relapsed after treatment, positive PCR results on peripheral blood almost always appeared before the clinical onset of disease (Lachaud *et al.* 2000; Pizzuto *et al.* 2001). Persistent infection in apparently healed scars has been reported for MCL (Delgado *et al.* 1996; Schubach *et al.* 1998). Guevara *et al.* (1994) reported the presence of *Leishmania braziliensis* in patients cured by immunotherapy or at different stages of treatment. They also found the parasite in subjects who had never suffered from leishmaniasis but who had lived in endemic areas and migrated to non-endemic regions many years earlier.

So far, only one report was published on the use of real-time PCR, namely for the quantification of *Leishmania* parasites in mouse liver (Bretagne *et al.* 2001). In the near future, this technique may play an increasingly important role in the quantification of promastigotes or amastigotes present in clinical samples and in the (simultaneous) identification of the infecting species or strain.

It can be concluded that PCR is capable of detecting the *Leishmania* parasite in a variety of clinical samples and for all clinical manifestations of the disease. PCR has caused a revolution in the diagnosis of leishmaniasis. However, one has to keep in mind that the execution of this very sensitive technique requires precautions:

- the risk of contamination necessitates dedicated laboratory areas for mix preparation, sample preparation and amplification/detection;
- false positive results can be further prevented by using the uracil nucleotide glycosylase/dUridine tri-phosphate (UNG/dUTP system) (Longo *et al.* 1990);
- appropriate and sufficient positive and negative controls need to be included in each experiment to confirm the sensitivity and specificity of the technique.

Another important issue that, so far, has hardly been addressed is the standardization of PCR technology for the diagnosis of leishmaniasis. Most laboratories use 'in house' PCR methods that are based on different primer pairs and DNA targets; the *Leishmania* PCR does not exist (Lachaud *et al.* 2002). There are only few comparative studies available in the literature (Meredith *et al.* 1993; Reithinger *et al.* 2000; Lachaud *et al.* 2002) and it would be very valuable to establish a universal PCR for the diagnosis of leishmaniasis.

Nucleic acid sequence-based amplification (NASBA)

Although PCR certainly has proved its merit in detecting *Leishmania* parasites and the diagnosis of leishmaniasis, a disadvantage of this technique is the fact that it is based on the detection of parasite DNA, which may be present a long time after the parasite has been cleared. NASBA technology, for the amplification of specific RNA sequences, has proven to be a very sensitive and specific assay in diagnostic microbiology (Compton 1991). Tests have been developed for HIV, human papillomavirus, *mycobacteria* and *Plasmodium falciparum*. The technique has not yet been developed for leishmaniasis. NASBA has several advantages over PCR: it detects RNA in a background of DNA and may thus serve to measure viable parasites; the NASBA reaction is isothermal (a thermo-cycler is not required) and quick (90 min); it is specific and sensitive, as little as 10–100 target molecules in a sample can be amplified. Moreover, NASBA can be used for the accurate quantification of RNA levels, which allows the accurate quantification (i.e. determining the actual number) of the infectious agent (Schoone *et al.* 2000). Quantitative analysis of RNA levels after drug treatment could be a useful method to assess the efficacy of anti-*Leishmania* treatment.

Despite these advantages, NASBA is not widely used. This not only holds true for leishmaniasis, but with the exception of HIV/AIDS, for most other diseases where its value was proved (such as malaria, tuberculosis and leprosy) as well. This is probably because of the fact that PCR and RT-PCR already fill the niche where NASBA could be of value.

Control

Vaccination is considered to be the best option to develop an effective strategy in controlling leishmaniasis (Tesh 1995; Dye 1996). In the past, numerous antigens have been tested with variable success for protection against CL in *in vitro* and mouse models (Handman 2001), but there are currently no (commercially) available effective vaccines against *Leishmania* in routine use anywhere in the world. However, the general opinion prevails that a vaccine against leishmaniasis is feasible (Handman 2001). Furthermore, vaccines have the additional advantage of avoiding the problems encountered with drug resistance. The latter is an increasing problem in the control of leishmaniasis. The mechanisms underlying resistance are being elucidated and may result in the development of molecular tools for monitoring the development of drug resistance.

Vaccine development

Leishmaniasis control would greatly benefit from the development of an effective vaccine. Traditionally, vaccine development has mainly been focused on cutaneous leishmaniasis, as cured individuals are protected from further disease. However, despite many efforts, as recently extensively reviewed by Handman (2001), to date there are no effective vaccines against any form of leishmaniasis available. The development of vaccines has been hampered by several factors: the complex life cycle of *Leishmania* involving at least two hosts; the antigenic diversity of the parasite makes it difficult to identify potential protective antigens; experimental animal models used to evaluate potential vaccines are not representative for disease in humans and the lack of sufficient financial support to develop a vaccine for a relatively poor part of the world population. Notwithstanding these difficulties, several antigens have been identified that may be potential vaccine candidates and molecular biological techniques have made them available as recombinant antigens for second generation vaccines.

Recombinant antigens

It is outside the scope of this review to extensively present all potential protective recombinant antigens. However, recently the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) has organized a comparative study of several leading recombinant antigens as part of an effort to systematically test candidate antigens as potential vaccine candidates. A report (TDR/PRD/LEISH/VAC/01.1) on this study can be downloaded from: www.who.int/tdr/publications/publications/leishmaniasis-vaccines.htm. The study comprised different recombinant *L. major*, *L. braziliensis* and *L. mexicana* antigens expressed in *Escherichia coli* either as a single recombinant protein or as a mixture. The evaluation consisted of: (1) testing the antigens for human peripheral blood mononuclear cell (PBMC) proliferation and (2) challenge experiments in immunized mice. Mice were immunized with two doses of 25 μg of recombinant antigen plus either recombinant interleukin 12 (rIL-12) or monophospholipid A as adjuvant.

Peripheral blood mononuclear cell stimulation experiments showed that most antigens induced some proliferative responses and interferon-gamma production, suggesting that the selected antigens have some degree of immunogenicity in humans. In contrast, none of the antigens produced significant protection in the mouse challenge experiments. These antigens were either used as a single recombinant protein, i.e. LACK (*Leishmania* homologueue of receptors for activated C kinase) and 4H6 (a *L. major* antigen), or as a mixture containing several antigens like heat shock proteins of *L. braziliensis*, *L. major* leishmanolysin (GP63) and LACK. Some of the tested antigens, irrespective of which type of adjuvant was used, showed some protection against *L. major* challenge in BALB/c mice in one laboratory, but not in another. Because of these conflicting results it is difficult to draw definitive conclusions from these experiments and the usefulness of these antigens as a vaccine in humans remains to be elucidated.

DNA vaccination

DNA vaccination may be a new approach for preventing infectious diseases for which conventional vaccines have failed (Ulmer *et al.* 1996; Wahren 1996). In DNA vaccination plasmid DNA containing the gene encoding the vaccine candidate and necessary regulatory elements to express it is introduced into the tissue via intramuscular injection or particle bombardment. Once the DNA reaches the tissue, it is taken up by cells and translocated to the nucleus, where it is transcribed into RNA and subsequently translated in the cytoplasm. Although the uptake and expression in DNA must be very low, the antigen is expressed in enough quantity to induce a potent and specific immune response and to confer protection against further infections (Hassan *et al.* 1999). The effectiveness of DNA vaccines against infectious agents has been demonstrated in numerous animal models, including a murine model for leishmaniasis in which the effectiveness of a GP63-based DNA vaccine was evaluated (Walker *et al.* 1998). In addition, vaccination with DNA encoding LACK

afforded protection to mice against infection with *L. major* (Gurunathan *et al.* 1997). The amino acid sequence of the *Leishmania* LACK antigen is highly conserved, but the efficacy of this vaccine antigen in preventing disease caused by strains other than *L. major* could not be demonstrated. DNA vaccination studies with a *L. donovani* LACK DNA vaccine, although inducing a robust parasite-specific Th1 immune response (IFN-gamma but not IL-4 production) and priming for an *in vivo* T-cell response to inoculated parasites, did not induce protection against cutaneous or systemic *L. donovani* challenge in mice (Melby *et al.* 2001).

DNA vaccination certainly holds great promise for the future of vaccine development against infectious diseases, especially in developing countries. Such vaccines are attractive because they generate appropriate immune responses, ensure correct folding of the antigen, do not require adjuvants, are simple to produce, which may make them affordable and stable and do not require a strict cold chain for distribution (Handman 2001). Furthermore, DNA vaccines are most likely to induce long-term cellular immunity (Gurunathan *et al.* 2000). However, transition of DNA vaccines from the laboratory to clinical studies in humans raises important safety issues, such as the integration of the vaccine DNA in the human genome and the induction of auto-immune disease, which must be appropriately addressed before any DNA vaccine becomes reality (Klinman *et al.* 2000; Mor & Eliza 2001).

Drug resistance

Because of the fact that an effective vaccine against leishmaniasis is not available, chemotherapy is the only effective way to treat all forms of disease. Throughout the world, pentavalent antimony containing drugs have been the first-line treatment of choice for more than 50 years. However, the use of these drugs is becoming limited by the emergence of drug resistance. Primary resistance or unresponsiveness to antimonials is found in approximately 1% of all previously untreated patients (Ben Salah *et al.* 2000); and in some cases relapse after initial response occurs (secondary resistance or unresponsiveness). The occurrence of drug resistant *Leishmania* strains is increasing, which is most evident in India, where in hyperendemic districts of north Bihar, 50–65% of the patients fail to respond to treatment with pentavalent antimonial compounds (Sundar *et al.* 2000). Important reasons for this increase are rampant use of subtherapeutic doses, incomplete duration of treatment and substandard drugs (Sundar 2001).

Understanding drug resistance is crucial for the future control of leishmaniasis. In contrast to the malaria situation, where for example point mutations in two genes (dihydrofolate reductase and dihydropteroate synthetase) confer the occurrence of resistance to the antimalaria drug sulfadoxine-pyrimethamine (Fansidar), the mechanisms underlying drug resistance in leishmaniasis are just beginning to be understood (Croft 2001; Ouellette 2001). This hampers the development of molecular probes or PCR-based diagnostics to monitor the development and spread of antileishmanial drug resistance.

Recent insights suggest that drug resistance comprises at least the following features. Firstly, the active form of pentavalent antimony is the trivalent form of the metal. The reduction of the pentavalent compound to a trivalent metal may take place either in the host macrophage (Sereno *et al.* 1998) or in the parasite (Shaked-Mishan *et al.* 2000). If the latter is the case, reduction or loss of reductase activity may lead to drug resistance. This hypothesis is supported by the observation that pentavalent antimony resistant *L. donovani* amastigotes lost their reductase activity (Shaked-Mishan *et al.* 2000). Secondly, increased levels of trypanothione (TSH), the major reduced thiol of *Leishmania* cells, are found in cells selected for resistance to trivalent antimony (Haimeur *et al.* 2000). This increase is caused by amplification of the γ-glutamylcysteine synthase (*GHS1*) gene (Grondin *et al.* 1997) and overexpression of the ornithine decarboxylase (*ODC*) gene (Haimeur *et al.* 1999), which are both involved in TSH biosynthesis. It is possible to reverse antimonial drug resistance by using TSH biosynthesis inhibitors. On the other hand, however, increasing TSH biosynthesis in wild type cells by transfecting *GSH1* or *ODC* does not lead to resistance, suggesting that high TSH levels are essential but not sufficient for resistance (Grondin *et al.* 1997; Haimeur *et al.* 1999). Thirdly, transport experiments indicate that a high level of drug resistance is mediated by an active extrusion system, an ATP-dependent transport system in membrane vesicles of *L. tarentolae* (Dey *et al.* 1996). Independently of this, the *Leishmania* ATP-binding cassette (ABC) transporter p-glycoprotein A (PGPA) is also involved in metal resistance, although the exact mechanism by which PGPA confers resistance to antimonials is not fully known (Ouellette *et al.* 1990). Co-transfection experiments, transport assays, and the use of inhibitors suggest that PGPA recognizes metals conjugated to glutathione or TSH. Localization studies indicate that PGPA is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite (Legare *et al.* 2001). Transport experiments using radioactive conjugates clearly showed that PGPA recognizes and actively transports thiol–metal conjugates. These results suggest that PGPA confers resistance by sequestrating the metal–thiol conjugates (Legare *et al.* 2001).

Most of the molecular and biochemical mechanisms underlying drug resistance elucidated so far are determined *in vitro* using promastigotes. It remains to be established whether comparable mechanisms are present in the amastigote stage of the parasite. There is a long way to go to identify a suitable *in vivo* amastigote marker for drug resistance.

Identification

Leishmania transmission cycle and epidemiology depend on the particular infecting (sub)species. Therefore, exact identification of the parasite species causing the disease is important in order to design the correct control strategy and to make decisions regarding treatment strategies. *Leishmania* parasites have been divided into different species primarily on the basis of clinical, biological, geographical and epidemiological criteria. During the past two decades, intrinsic characteristics, such as biochemical and molecular data, have been used for the classification of *Leishmania* isolates. At present, isoenzyme analysis by electrophoresis is widely used for the identification of *Leishmania* (sub)species and up to now it still represents the reference technique for *Leishmania* identification. The parasites can be identified by their enzymatic profile and be grouped in taxonomic units termed zymodemes. However, isoenzyme analysis is time consuming and laborious, requires culturing and obtaining the profile of 10–20 different enzymes. Molecular methods, such as Southern hybridization and PCR-based methods have become available that are less laborious and more powerful to study variability between *Leishmania* species (Morales *et al*. 2001).

Southern blotting using DNA probes

One of the first molecular characterization methods in use was Southern blotting using DNA probes. This method is tedious as it requires cultivation of promastigotes, DNA extraction, gel electrophoresis, Southern blotting and hybridization (Van Eys *et al*. 1989). DNA probes for identifying *Leishmania* spp. generally target kinetoplast DNA (kDNA), because the kDNA minicircle molecules are present at 10 000 copies and have a variable region that differs from minicircle classes in the same network (Barker 1989). kDNA minicircle sequence probes have been developed for *L. major* (Smith *et al*. 1989), dermatropic and viscerotropic strains of *L. infantum* (Gramiccia *et al*. 1992) and *L. aethiopica* (Laskay *et al*. 1991). Recently, a new minicircle class exclusive to *Leishmania* (*Viannia*) *guyanensis* was identified (Rodriguez *et al*. 2000). This allowed the development of a specific probe, which hybridized strongly only to *L.* (*V.*) *guyanensis* kDNA after medium stringency washing.

Probes derived from different sequences of the nuclear DNA have also been described. A cDNA probe containing multiple copies of a 60-bp repetitive degenerate sequence isolated from *L. donovani* specifically hybridized only with isolates of the *L. donovani* complex (Howard *et al*. 1991). Van Eys *et al*. (1989, 1991) described two probes which could be used for the differentiation of *Leishmania* species. One probe, pDK10, can be used to distinguish the Old World CL causing species from *L. donovani* complex and the other, pDK20, to differentiate between all Old World *Leishmania* species.

PCR-based techniques for identification

Many PCR-based techniques have been developed for the identification of *Leishmania* species.

Species specific primers: the specificity of any given PCR assay can be adapted to specific needs by targeting conserved or variable regions in parasite DNA. In this way it is possible to characterize *Leishmania* to the level of the genus complex, species or even the individual isolate. For example, PCR amplification of mini-exon gene repeating units showed size and sequence variation amongst many *Leishmania* species (Ramos *et al*. 1996), especially in the non-transcribed spacer region, which is distinct in length and in sequence among different *Leishmania* species (Katakura *et al*. 1998).

Random amplified polymorphic DNA (RAPD) uses a single primer of arbitrary sequence at a low annealing temperature to produce a PCR fingerprint-banding pattern after gel electrophoresis. This method does not depend upon previous knowledge or availability of the nucleotide sequence of the target nor does it require DNA hybridization (Welsh & McCleland 1990). However, the RAPD method has one major drawback: it can only be used on cultured parasites free of contaminating host DNA, which would mask the signal from the parasite DNA (Noyes *et al*. 1996). A correlation between RAPD pattern and isoenzyme analysis results was observed among different *Leishmania* isolates using six different arbitrary primers (Tibayrenc *et al*. 1993). Four different, closely related *Leishmania* spp. from the Viannia group were used to evaluate 28 different RAPD primers. Thirteen of these primers yielded reproducible multiple banding patterns of taxonomic value (Noyes *et al*. 1996).

Amplification of genomic DNA with primers annealing to mini- and microsatellite DNA sequences yielded distinctive and reproducible sets of amplified DNA fragments

for all *Leishmania* isolates tested. The number and size of amplification products were found to be characteristic for a given species. By comparing PCR patterns of unidentified *Leishmania* isolates with those obtained from reference strains it is possible to identify these isolates at the species level (Schönian *et al*. 1996).

Restriction fragment length polymorphism (RFLP): with RFLP a PCR amplicon is digested with a set of different restriction enzymes and resulting fragments are separated according to molecular size using gel electrophoresis. For example, by digesting the amplified small subunit ribosomal RNA PCR product with the restriction enzyme *Rsa*I, it is possible to distinguish *L. donovani* from *L. tropica* and *L. major* (Van Eys *et al*. 1992).

The GP63 locus has been successfully used for the genetic characterization of a large number of natural isolates belonging to four species of the subgenus *Viannia*, namely *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *lainsoni* (Victoir *et al*. 1998). Cupolillo *et al*. (1995) has exploited the variability of the transcribed non-coding regions between the small and large subunit rRNA genes to examine relationships in the *Viannia* subgenus. In a method termed intergenic region typing (IRT), PCR amplification products were obtained for the rapidly evolving 1–1.2-kb internal transcribed spacers (ITS) between the SSU and LSU rRNAs, from 50 parasites isolated from different hosts and geographical areas. Amplified DNAs were cut with different restriction enzymes, and fragment patterns compared after acrylamide gel electrophoresis. High levels of intra- and interspecific variation were observed, and quantitative similarity comparisons were used to associate different lineages. A complex evolutionary tree was obtained. Some species formed tight clusters (*L. equatorensis*, *L. panamensis*, *L. guyanensis*, *L. shawi*), while *L. braziliensis* was highly polymorphic and *L. naiffi* showed intraspecific distances comparable to the largest obtained within all *Viannia*. *L. colombiensis*, *L. equatorensis* and *L. lainsoni* clearly represent distinct lineages. Good agreement was obtained with molecular trees based upon isoenzyme or mini-exon repeat sequence comparisons.

Single strand conformation polymorphism (SSCP) analysis is based on the differences in the secondary structure of single-strand DNA molecules differing in a single nucleotide, which also is frequently reflected in an alteration of their electrophoretic mobility in non-denaturing gel electrophoresis. SSCP analysis was able to detect genetic diversity at the level of a single nucleotide in a set of Sudanese *L. donovani* isolates (El Tai *et al*. 2001) and in 29 *L. tropica* strains (Schönian *et al*. 2001).

Molecular techniques and taxonomy

Molecular techniques have provided new or improved views with regard to a number of taxonomic matters:

So far no differences were found between *L. infantum* and *L. chagasi*, supporting the idea that *L. chagasi* and *L. infantum* are identical and that this species was introduced in the New World in the recent past (Mauricio *et al*. 2000), maybe as part of the Spanish and Portuguese conquest of Latin America.

In another matter, the question whether *Leishmania* is a sexually or asexually reproducing organism, the current consensus seems to be that *Leishmania* has a basically clonal population structure with occasional bouts of genetic exchange or hybridization (Tibayrenc & Ayala 1999).

RFLP data suggest that the Old World *Leishmanias* comprise a monophyletic lineage, with species associated with cutaneous disease exhibiting the greatest level of divergence (Pogue *et al*. 1996).

However, a number of taxonomic questions with regard to *Leishmania* remain open to debate. These are related to the *Viannia* subgenus and the taxonomic status of a number of New World complexes and have partly been addressed by Cupolillo *et al*. (2001).

New developments

Genome sequencing project

The *Leishmania* genome is a relatively small eukaryotic genome with an estimated size of 33.6 Mbp with a karyotype of 36 chromosomes, ranging in size from 0.3 to 2.5 Mbp (Sunkin *et al*. 2000). In imitation of other genome sequencing projects, the Leishmania Genome Network (LGN) was established in Rio de Janeiro (Brazil) in 1994 and aimed at the genomic sequencing of the reference strain *Leishmania major* Friedlin (Ivens & Blackwell 1999). The LGN is chaired by Professor J. M. Blackwell (Cambridge, UK) and 10 laboratories from seven countries are working for this project. The LGN is a programme supported by the UNDP/WORLD BANK/WHO – TDR and its website www.ebi.ac.uk/parasites/leish.html is a important source for *Leishmania* sequence information, general information on parasites and their genomes and allows access to valuable bioinformatic tools via its links.

The aim of the LGN was to obtain a detailed high-resolution map of the reference strain *L. major* Friedlin (MHOM/IL/81/Friedlin). This has been achieved by the application of a number of complementary approaches: the determination of a pulsed field gel (PFG)

chromosomal 'karyotype', shuttle cosmid clone fingerprinting to generate overlapping contigs, sequencing and mapping of expressed sequence tags (ESTs) to PFG-separated chromosomes and the generation of DNA sequence from entire chromosomes. Sequencing of the whole genome is in progress. A first-generation cosmid contig map of *L. major* Friedlin genome has been constructed and sequencing of the genome is well under way with chromosome 1 (Chr1) and Chr2 completed and Chr4 virtually complete (Myler & Stuart 2000). Furthermore, active sequencing of Chr19, 23 and 35 is underway (LGN web site 2001).

More than 600 completely sequenced genes have been identified, an estimated 8% of the genome of approximately 8600 genes of *Leishmania*. Up to date, a large portion of the newly identified genes (> 60%) remains unclassified, with 40% of these being potentially *Leishmania* specific.

Chr1, the smallest chromosome of *L. major* Friedlin, consists of three homologues which differ in size by approximately 29 kb. The complete sequence of Chr1 predicts that this chromosome has 79 protein coding sequences. The first 29 of these genes are encoded in tandem on one strand of DNA, and the remaining 50 genes are encoded on the other (McDonagh *et al.* 2000). No RNA polymerase promoters, centromeric sequences of origins of DNA replication have been identified in the DNA sequence. Statistical analysis of Chr1 showed that the divergent junction region between the two polycistronic gene clusters may be a candidate for an origin of DNA replication (Myler *et al.* 2000).

In the coming years, the LGN will shift into the next phase: functional genomics and proteomics. This emerging area of research in the 'post-genomic era' deals with the global analysis of gene expression using a battery of techniques to resolve (by 2D PAGE), identify (by peptide sequencing, mass spectrometry, immunoblotting, etc.), quantify and characterize proteins, as well as to store and interlink protein and DNA sequence and mapping information from the genome projects. This will aid identification of the molecular determinants of virulence in *Leishmania* and new strategies that can be exploited in the search for new therapies and vaccines.

Microarrays

The biological functions of only a fraction of the genes that are identified in genome projects are known. Traditional molecular biological methods work on one gene – one experiment basis and because of the limited throughput of such methods, the whole picture of gene function is difficult to obtain. In contrast, the recently developed microarray technology, in which DNA molecules representing many genes are placed in thousands of discrete spots on a microscope slide, allows scientists to look at many genes at once and to determine which ones are expressed in a particular cell type. Total mRNA from cells in two different conditions is extracted and labelled with two different fluorescent labels: for example, a green dye for cells at condition 1 (e.g. the *Leishmania* promastigote stage) and a red dye for cells at condition 2 (amastigote stage). Both extracts are washed over the microarray. Labelled gene products from the extracts hybridize to their complementary sequences in the spots because of base-pairing. The fluorescent dyes enable measuring the amount of sample. If the RNA from the promastigote is in abundance, the spot will be green, if the RNA from the amastigote, it will be red. If both are equal, the spot will be yellow, while if neither is present the spot will appear black. Thus, from the fluorescence intensities and colours for each spot, the relative translation levels of the genes in both samples can be estimated. More background information on DNA array technology can be found in Anthony *et al.* (2001), including several references to relevant websites.

It is expected that the application of microarray technology in combination with the *Leishmania* genome project will allow the identification of many new drug and vaccine targets. Recently, Professor J.M. Blackwell reported at the Fourth TDR/IDRI meeting on second generation vaccines against leishmaniasis on the use of microarrays to identify potential new vaccine targets. DNA microarrays were used to simultaneously monitor the expression profiles for 2183 unique *Leishmania* genes as the parasite undergoes developmental transition from the logarithmic promastigote to the metacyclic form, and in the host-derived amastigote form. From this analysis, more than 100 previously unknown genes were identified that are up-regulated in expression in amastigotes. These are now being tested as new vaccine candidates; some cocktails of them appear to be effective as DNA vaccines in mice.

Concluding remarks

The field of molecular biology is developing rapidly and genome projects hold great promise for the future development of tools that can be used for the diagnosis and control of infectious diseases. These developments must be exploited in full in *Leishmania* research as we are still facing major problems, e.g. lack of an effective vaccine and the development of drug resistance, in controlling leishmaniasis. In addition, new technology may become available for the diagnosis of the disease in particular for HIV/*Leishmania* coinfections.

References

Adhya S, Chatterjee M, Hassan MQ, Mukherjee S & Sen S (1995) Detection of *Leishmania* the blood of early kala-azar patients with the aid of the polymerase chain reaction. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **89**, 622–624.

Andresen K, Gaafar A, El-Hassan AM *et al.* (1996) Evaluation of the polymerase chain reaction in the diagnosis of cutaneous leishmaniasis due to *Leishmania major*: a comparison with direct microscopy of smears and sections from lesions. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **90**, 133–135.

Andresen K, Gasim S, Elhassan AM *et al.* (1997) Diagnosis of visceral leishmaniasis by the polymerase chain reaction using blood, bone marrow and lymph node samples from patients from the Sudan. *Tropical Medicine and International Health* **2**, 440–444.

Anthony RM, Brown TJ & French GL (2001) DNA array technology and diagnostic microbiology. *Expert Reviews on Molecular Diagnostics* **1**, 15–23.

Aviles H, Belli A, Armijos R, Monroy FP & Harris E (1999) PCR detection and identification of *Leishmania* parasites in clinical specimens in Ecuador: a comparison with classical diagnostic methods. *Journal of Parasitology* **85**, 181–187.

Barker DC (1989) Molecular approaches to DNA diagnosis. *Parasitology* **99**, S125–S146.

Ben Salah AB, Ben Ismail R, Amri F *et al.* (2000) Investigation of spread of human visceral leishmaniasis in central Tunisia. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **94**, 382–386.

Bretagne S, Durand R, Olivi M *et al.* (2001) Real-time PCR as a new tool for quantifying *Leishmania infantum* liver in infected mice. *Clinical and Diagnostic Laboratory Immunology* **8**, 828–831.

Burns JM, Shreffler WG, Benson DR, Ghalib HW, Badaro R & Reed SG (1993) Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. *Proceedings of the National Academy of Sciences of the USA* **15**, 775–779.

Campino L, Cortes S, Pires R, Oskam L & Abranches P (2000) Detection of *Leishmania* immunocompromised patients using peripheral blood spots on filter paper and the polymerase chain reaction. *European Journal of Clinical Microbiology and Infectious Diseases* **19**, 396–398.

Compton J (1991) Nucleic acid sequence-based amplification. *Nature* **250**, 91–92.

Croft SL (2001) Monitoring drug resistance in leishmaniasis. *Tropical Medicine and International Health* **6**, 899–905.

Cupolillo E, Grimaldi G, Momen H & Beverly SM (1995) Intergenic region typing (IRT): a rapid molecular approach to the characterization and evoluation of *Leishmania*. *Molecular and Biochemical Parasitology* **73**, 145–155.

Cupolillo E, Aguiar Alves F, Brahim LR *et al.* (2001) Recent advances in the taxonomy of the New World leishmanial parasites. *Medical Microbiology and Immunology* **190**, 57–60.

Delgado O, Guevara P, Silva S, Belfort E & Ramirez JL (1996) Follow-up of a human accidental infection by *Leishmania braziliensis* using conventional immunologic techniques and polymerase chain reaction. *American Journal for Tropical Medicine and Hygiene* **55**, 267–272.

Dey S, Ouellette M, Lightbody J, Papadopoulou B & Rosen BP (1996) An ATP-dependent As(III) -glutathione transport system in membrane vesicles of *Leishmania tarentolae*. *Proceedings of the National Academy of Sciences of the USA* **93**, 2192–2197.

Dye C (1996) The logic of visceral leishmaniasis control. *American Journal of Tropical Medicine and Hygiene* **55**, 125–130.

El Tai NO, El Fari M, Mauricio I *et al.* (2001) *Leishmania donovani*: intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. *Experimental Parasitology* **97**, 35–44.

Gramiccia M, Smith DF, Angelici MC, Ready PD & Gradoni L (1992) A kinetoplast DNA probe diagnostic for *Leishmania infantum*. *Parasitology* **105**, 29–34.

Grondin K, Haimeur A, Mukhopadyay R, Rosen BP & Oullette M (1997) Co-amplification of the gamma-glutamylcysteine synthetase gene *gsh1* and of the ABC transporter gene *pgpA*. arsenite-resistant *Leishmania tarentolae*. *EMBO Journal* **16**, 3057–3065.

Guevara P, Rojas E, Gonzalez N *et al.* (1994) Presence of *Leishmania braziliensis*. blood samples from cured patients or at different stages of immunotherapy. *Clinical Diagnostic and Laboratory Immunology* **1**, 385–389.

Gurunathan S, Sacks DL, Brown DR *et al.* (1997) Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *Journal of Experimental Medicine* **186**, 1137–1147.

Gurunathan S, Wu C, Freidag BL & Seder RA (2000) DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinions in Immunology* **12**, 442–447.

Haimeur A, Brochu C, Genest P, Papadopoulou B & Oullette M (2000) Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. *Molecular and Biochemical Parasitology* **108**, 131–135.

Haimeur A, Guimond C, Pilote S *et al.* (1999) Elevated levels of polyamines and trypanothione resulting from overexpression of ornithine decarboxylase gene in arsenite-resistant *Leishmania*. *Molecular Microbiology* **34**, 726–735.

Handman E (2001) Leishmaniasis: current status of vaccine development. *Clinical Microbiology Reviews* **14**, 229–243.

Hassan UA, Abai AM, Harper DR, Wren BW & Morrow WJ (1999) Nucleic acid immunization: concepts and techniques associated with third generation vaccines. *Journal of Immunological Methods* **229**, 1–22.

Houghton RL, Petrescu M, Benson DR *et al.* (1998) A cloned antigen (recombinant K39) of *Leishmania chagasi* diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. *Journal of Infectious Diseases* **177**, 1339–1344.

Howard MK, Kelly JM, Lane RP & Miles MA (1991) A sensitive repetitive DNA probe that is specific to the *Leishmania donovani* complex and its use as an epidemiological and diagnostic reagent. *Molecular and Biochemical Parasitology* **44**, 63–72.

Ivens AC & Blackwell JM (1999) The *Leishmania* genome comes of age. *Parasitology Today* **15**, 225–231.

Jelinek T, Eichenlaub S & Loscher T (1999) Sensitivity and specificity of a rapid immunochromatographic test for diagnosis of visceral leishmaniasis. *European Journal of Clinical Microbiology and Infectious Diseases* **18**, 669–670.

Katakura K, Kawazu SI, Sanjyoba C *et al.* (1998) *Leishmania* mini-exon genes for molecular epidemiology of leishmaniasis in China and Ecuador. *Tokai Journal of Experimental Clinical Medicine* **23**, 393–399.

Klinman DM, Takeshita F, Kamstrup S *et al.* (2000) DNA vaccines: capacity to induce auto-immunity and tolerance. *Developmental Biology* **104**, 45–51.

Lachaud L, Dereure J, Chabbert E *et al.* (2000) Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. *Journal of Clinical Microbiology* **38**, 236–240.

Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP & Bastien P (2002) Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *Journal of Clinical Microbiology* **40**, 210–215.

Laskay T, Kiessling R, de Witt RTF & Wirth DF (1991) Generation of species-specific DNA probes for *Leishmania aethiopica*. *Molecular and Biochemical Parasitology* **44**, 279–286.

Legare D, Cayer S, Singh AK, Richard D, Papadopoulou B & Oullette M (2001) ABC proteins of *Leishmania*. *Journal of Bioenergetics and Biomembranes* **6**, 469–474.

Longo MC, Berninger MS & Hartley JL (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. *Gene* **93**, 125–128.

Mathis A & Deplazes P (1995) PCR and *in vitro* cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *Journal of Clinical Microbiology* **33**, 1145–1149.

Mauricio IL, Stothard JR & Miles MA (2000) The strange case of *Leishmania chagasi*. *Parasitology Today* **16**, 188–189.

McDonagh PD, Myler PJ & Stuart K (2000) The unusual gene organization of *Leishmania major* chromosome 1 may reflect novel transcription processes. *Nucleic Acids Research* **15**, 2800–2803.

Melby PC, Yang J, Zhao W, Perez LE & Cheng J (2001) *Leishmania donovani* p36 (LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Infection and Immunity* **69**, 4719–4725.

Meredith SEO, Zijlstra EE, Schoone GJ *et al.* (1993) Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. *Archives de L'Institut Pasteur de Tunis* **70**, 419–431.

Mor G & Eliza M (2001) Plasmid DNA vaccines. Immunology, tolerance and autoimmunity. *Molecular Biotechnology* **19**, 245–250.

Morales MA, Chicharro C, Ares M, Canavate C, Barker DC & Alvar J (2001) Molecular tracking of infections by *Leishmania infantum*. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **95**, 104–107.

Myler PJ, Sisk E, McDonagh PD *et al.* (2000) Genomic organization and gene function in *Leishmania*. *Biochemical Society Transactions* **28**, 527–531.

Myler PJ & Stuart KD (2000) Recent developments from the *Leishmania* genome project. *Current Opinions in Microbiology* **3**, 412–416.

Noyes HA, Beli AA & Maingon R (1996) Appraisal of various random amplified polymorphic DNA-polymerase chain reaction primers for *Leishmania* identification. *American Journal of Tropical Medicine and Hygiene* **55**, 98–105.

Nuzum E, White F, Thakur C *et al.* (1995) Diagnosis of symptomatic visceral leishmaniasis by use of the polymerase chain reaction on patient blood. *Journal of Infectious Diseases* **171**, 751–754.

Osman OF (1998) *Visceral Leishmaniasis: the PCR and Direct Agglutination Test for Diagnosis and Management*. PhD Thesis, University of Amsterdam, The Netherlands.

Osman OF, Oskam L, Zijlstra EE *et al.* (1997a) Evaluation of PCR for diagnosis of visceral leishmaniasis. *Journal of Clinical Microbiology* **35**, 2454–2457.

Osman OF, Kager PA, Zijlstra EE, el-Hassan AM & Oskam L (1997b) Use of PCR on lymph-node samples as test of cure of visceral leishmaniasis. *Annals of Tropical Medicine and Parasitology* **91**, 845–850.

Osman OF, Oskam L, Zijlstra EE, el-Hassan AM, el-Naeim DA & Kager PA (1998) Use of the polymerase chain reaction to assess the success of visceral leishmaniasis treatment. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **92**, 397–400.

Ouellette M (2001) Biochemical and molecular mechanisms of drug resistance in parasites. *Tropical Medicine and International Health* **6**, 874–882.

Ouellette M, Fase-Fowler F & Borst P (1990) The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. *EMBO Journal* **9**, 1027–1033.

Piarroux R, Gamarelli F, Duman H *et al.* (1994) Comparison of PCR with direct examination of bone marrow aspiration, myeloculture, and serology for diagnosis of visceral leishmaniasis in immunocompromised patients. *Journal of Clinical Microbiology* **32**, 649–749.

Pirmez C, da Silva Trajano V, Paes-Oliveira Neto M *et al.* (1999) Use of PCR in diagnosis of human American tegumentary leishmaniasis in Rio de Janeiro, Brazil. *Journal of Clinical Microbiology* **37**, 1819–1823.

Pizzuto M, Piazza M, Senese D *et al.* (2001) Role of PCR in diagnosis and prognosis of visceral leishmaniasis in patients co-infected with human immunodeficiency virus type 1. *Journal of Clinical Microbiology* **39**, 357–361.

Pogue GP, Joshi M, Lee NS *et al.* (1996) Conservation of low-copy gene loci in Old World Leishmanias identifies mechanisms of parasite evolution and diagnostic markers. *Molecular and Biochemical Parasitology* **18**, 27–40.

Qu JQ, Zhong L, Masoom-Yasinzai M *et al.* (1994) Serodiagnosis of Asian leishmaniasis with a recombinant antigen from the repetitive domain of a *Leishmania* kinesin. *Transactions of*

the Royal Society for Tropical Medicine and Hygiene **88**, 543–545.

Ramos A, Maslov DA, Fernandes O, Campbell DA & Simpson L (1996) Detection and identification of human pathogenic *Leishmania* and *Trypanosoma* species by hybridization of PCR-amplified mini-exon repeats. *Experimental Parasitology* **82**, 242–250.

Reithinger R, Lambson BE, Barker DC & Davies CR (2000) Use of PCR to detect *Leishmania* (Viannia) spp. in dog blood and bone marrow. *Journal of Clinical Microbiology* **38**, 748–751.

Rodriguez N, Rodriguez A, Cardona M, Barrios MA, McCann SH & Barker DC (2000) *Leishmania (Viannia) guyanensis*: a new minicricle class exclusive to this species isolated from a DNA cosmid library useful for taxonomic purposes. *Experimental Parasitology* **94**, 143–149.

Salotra P, Sreenivas G, Pogue GP *et al*. (2001) Development of a species-specific PCR assay for detection of *Leishmania donovani*. clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. *Journal of Clinical Microbiology* **39**, 849–854.

Schallig HDFH, Schoone GJ, Kroon CCM, Hailu A, Chappuis F & Veeken H (2001) Development and application of 'simple' diagnostic tools for visceral leishmaniasis. *Medical Microbiology and Immunology* **190**, 69–71.

Schönian G, Schweynoch C, Zlateva K *et al*. (1996) Identification and determination of the relationships of species and strains within the genus *Leishmania* using single primers in the polymerase chain reaction. *Molecular and Biochemical Parasitology* **77**, 19–29.

Schönian G, Schnur L, el Fari M *et al*. (2001) Genetic heterogenicity in the species *Leishmania tropica* revealed by different PCR-based methods. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **95**, 217–224.

Schoone GJ, Oskam L, Kroon NCM, Schallig HDFH & Omar SA (2000) Detection and quantification of *Plasmodium falciparum*. blood samples using quantitative nucleic acid sequence-based amplification. *Journal of Clinical Microbiology* **38**, 4072–4075.

Schubach A, Haddad F & Oliveira-Neto MP (1998) Detection of leishmania DNA by polymerase chain reaction in scars of treated human patients. *Journal of Infectious Diseases* **178**, 911–914.

Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouissi A & Lemesre JL (1998) Axenically grown amastigotes of *Leishmania infantum* used as an *in vitro* model to investigate the pentavalent antimony mode of action. *Antimicrobial Agents and Chemotherapy* **42**, 3097–3102.

Shaked-Mishan P, Ulrich N, Ephros M & Zilberstein D (2000) Novel intracellular Sb(V) reducing activity correlates with antimony susceptibility in *Leishmania donovani*. *Journal of Biological Chemistry* **276**, 3971–3976.

Smith DF, Searle S, Ready PD, Gramiccia M & Ben-Ismail R (1989) A kinetoplast DNA probe diagnostic for *Leishmania major*: sequence homologies between regions of *Leishmania* minicircles. *Molecular and Biochemical Parasitology* **37**, 213–224.

Sundar S (2001) Drug resistance in Indian visceral leishmaniasis. *Tropical Medicine and International Health* **6**, 849–854.

Sundar S, More DK, Singh MK *et al*. (2000) Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clinical Infectious Diseases* **31**, 1104–1107.

Sunkin SM, Kiser P, Myler PJ & Stuart K (2000) The size difference between *Leishmania major* Friedlin chromosome one homologues is localized to sub-telomeric repeats at one chromosomal end. *Molecular and Biochemical Parasitology* **109**, 1–15.

Tesh RB (1995) Control of zoonotic visceral leishmaniasis: is it time to change strategies? *American Journal of Tropical Medicine and Hygiene* **52**, 287–292.

Tibayrenc M & Ayala FJ (1999) Evolutionary genetics of *Trypanosoma* and *Leishmania*. *Microbes and Infection* **1**, 465–472.

Tibayrenc M, Neubauer K, Barnabe C, Guerrini F, Skarecky D & Ayala FJ (1993) Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proceedings of the National Academy of Sciences of the USA* **90**, 1335–1339.

Ulmer JB, Sadoff JC & Liu MA (1996) DNA vaccines. *Current Opinions in Immunology* **8**, 531–536.

Van Eys GJJM, Schoone GJ, Ligthart GS, Alvar J, Evans DA & Terpstra WJ (1989) Identification of Old World *Leishmania* by DNA recombinant probes. *Molecular and Biochemical Parasitology* **34**, 53–62.

Van Eys GJJM, Guizani I, Ligthart GS & Dellagi K (1991) A nuclear probe for the identification of strains within the *Leishmania donovani* complex. *Experimental Parasitology* **72**, 459–463.

Van Eys GJJM, Schoone GJ, Kroon NCM & Ebeling SB (1992) Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. *Molecular and Biochemical Parasitology* **51**, 133–142.

Veeken H (2001) *Control of Infectious Disease in Developing Countries: Field Studies on Visceral Leishmaniasis and Meningitis*. PhD Thesis, University of Amsterdam, The Netherlands.

Victoir K, Banuls AL, Arevalo J *et al*. (1998) The *gp63* gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus Viannia. *Parasitology* **117**, 1–13.

Wahren B (1996) Gene vaccines. *Immunotechnology* **2**, 77–83.

Walker PS, Scharton-Kersten T, Rowton ED *et al*. (1998) Genetic immunization with glycoprotein 63 cDNA results in a T helper cell type 1 response and protection in a murine model of leishmaniasis. *Human Gene Therapy* **9**, 1899–1907.

Welsh J & McCleland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213–7218.

World Health Organization (2000) *Leishmania/HIV Co-Infection*. WHO/LEISH/2000.42, Geneva.

Zijlstra EE, Nur Y, Desjeux P, Khalil EA, El-Hassan AM & Groen J (2001) Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. *Tropical Medicine and International Health* **6**, 108–113.