Research Paper

A review: Identification and diagnosis of visceral leishmaniasis

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ABSTRACT

Leishmaniasis is a disease that ranges in severity from skin lesions to serious disfigurement and fatal systemic infection. World Health Organization (WHO) has classified the disease as emerging and uncontrolled. It is estimated that the infection results in two million new cases yearly. There are 12 million people currently infected worldwide and leishmaniasis threatens 350 million people in 88 countries. Leishmania parasites are divided into different species based primarily on clinical, biological, geographical and epidemiological criteria. In the Middle East and Central Asia, the predominant species responsible for leishmaniasis are L. major, L. tropica, L. donovani, L. braziliensis, L. mexicana, L. amazonensis, L. panamensis and L. infantum. In Pakistan, the disease has been highly endemic in tribal areas and described in its classic form and as variants of the classic variety. Globally, leishmaniasis is present in three different forms: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis and (iii) mucocutaneous leishmaniasis. After recovery, some patients (50% in Sudan and 1 to 3% in India) develop post kala-azar dermal leishmaniasis (PKDL), which requires prolonged and expensive treatment. PKDL patients also play an important role in VL transmission. VL is typically caused by the Leishmania donovani complex, which includes three species: L. donovani, Leishmania infantum and Leishmania chagasi. Leishmania transmission cycle and epidemiology depends on the particular infecting (sub) species, and each parasite has a unique epidemiological pattern. Therefore, exact identification and diagnosis of the parasite species causing the disease is important in order to design the correct control strategy and to make decisions regarding treatment strategies. Keeping aforementioned in view this review focuses on identification and diagnosis of visceral leishmaniasis.

Key words: Leishmaniasis, skin lesion, WHO, Pakistan, neglected disease.

INTRODUCTION

Leishmaniasis (a group of diseases) is caused by obligate intracellular protozoa of the genus Leishmania (Leishman, 1903; Sundar and Rai, 2002), transmitted by sandfly of the genus Phlebotomus (Old World) or Lutzomyia (New World) (Akhoundi et al., 2016).

Leishmaniasis, second most prevalent parasitic infection in the world after malaria, is a neglected disease or rather a disease that affects neglected or marginalized individuals. It dates back to the 7th century BC (Mursalin et al., 2015) and has been largely ignored due to its complex epidemiology and association with poverty (Mikhail and Dagne, 2014).

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. More than twenty (20) species having distinct epidemiological
patterns are responsible for Leishmania. Leishmania genome project accelerated the research in this field since the last decade.

Leishmaniasis is present in three different forms: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis, and (iii) mucocutaneous leishmaniasis. The visceral form, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia and hyper-gamma-globulinemia and is complicated by serious infections. It is the most severe form of the disease and left untreated is usually fatal.

Majority of cases occurs in India and its neighbors Bangladesh and Nepal, and Brazil and Sudan. Between 20 to 60% (depending on geographical location) of VL patients develop a syndrome known as post kala-azar dermal leishmaniasis (PKDL), which appears within a few years of the complete cure of VL. PKDL patients are considered a major source of parasites for new infections as a result of the large number of organisms in the skin accessible to sand fly bites (Kedzierski L, 2010). VL is also an important in Latin America, Africa, India, and southern Europe. Although a number of Leishmania species canvicseralize, three closely related ones account for the vast majority of cases of VL: L. donovani, L. infantum, and L. chagasi.

The diagnosis of VL is complex because its clinical features are shared by a host of other commonly occurring diseases, such as malaria, typhoid and tuberculosis; many of these diseases can be present along with VL (in cases of coinfection); sequestration of the parasite in the spleen, bone marrow, or lymph nodes further complicates this issue (Sundar and Rai, 2002).

In Pakistan, Leishmania has been highly endemic in all parts but is more prevalent in the hilly areas (Khan and Muneeb, 2011). CL cases are mostly reported, while VL is neglected in Pakistan. Therefore, this paper focuses on the identification and diagnosis of VL.

MATERIALS AND METHODS

Sample collection and preparation

The lesion (sample collection site) should be less than two (2) months old. Test was performed as soon as possible after collection of the sample. Samples were not left at room temperature for prolonged periods and refrigerated at 2 to 8°C for 24 h. Samples were stored below -20°C and brought to room temperature prior to testing. One of the precautions to be taken into consideration in the preparation process is that frozen samples should be completely thawed prior to testing and should not be repeatedly frozen and thawed.

Preparation of spiked samples

Spiking freshly drawn peripheral blood from a healthy volunteer is used through the hanging drop method. Pre-dilution of the parasites in 2 ml cRPMI media is made and a micro drop of it is put on the cavity using sterile glass micropipette. The slide is sealed with the cover slip and sealing media to prevent drying of the drop. Several dilutions were tested till a single parasite in the drop under an inverted microscope was seen which is then spiked into 200 μl of blood. DNA from these spiked samples can be isolated using any DNA Extraction Kit.

Identification and diagnosis

Diagnosis of Kala-azar is problematic; it can be fatal if not diagnosed on time. The spectrum of illness ranges from asymptomatic infection or self-resolving disease to fulminant, severe, life-threatening infection; many subclinical cases occur and go unrecognized.

The presumptive diagnosis is based on clinical findings (fever, hepatosplenomegaly and weight loss, etc), exclusion of other possible diagnoses, and response to pentavalent antimony therapy (Craig et al., 2017).

Treatment is lengthy and relatively expensive. The standard approach to the diagnosis of VL has been the identification of amastigotes in tissue or promastigotes in cultures of splenic or bone marrow aspirates while in some regions, lymphnodes are also sampled.

The diagnostic sensitivity of splenic aspiration is high (95 to 98%), while bone marrow specimens is lower (53 to 95%), respectively. Procedures used to obtain these specimens can be painful, and in the case of splenic aspiration, life-threatening hemorrhage occurred in rare instances. Specialized laboratory equipments and staffs are required to interpret results. Although not protective, high titers of anti-leishmanial antibodies are typical in patients with VL.

Culture or PCR testing of aspirate material improves parasitologic yield, but these methods are seldom undertaken outside of research laboratories (Mursalin et al., 2015). For Identification and diagnosis a number of tests were used in various places.

Direct microscopic examination

The amastigote forms of the parasite (called ‘LD bodies’) can be seen intracellularly in monocytes or macrophages on microscopic examination of Giemsa-stained blood or aspirates from lymph nodes, bone marrow or spleen. Amastigotes are round or oval bodies, 2 to 4 μm in diameter with characteristic organelles (nucleus and kinetoplast). Microscopy is done by preparation of biopsy sample. Smears are prepared, fixed in methanol and stained with Giemsa and examined under the microscope with a 40 X lens and with a 100 X oil immersion lens. If at least one intra- or extracellular amastigote with a distinctive kinetoplast is found the smear is declared positive. When
no amastigotes are seen after 15 min of inspection, the smear is declared negative. The patient's smears are double checked for confirmation (Rahi et al., 2015).

The sensitivity of direct microscopic examination varies, but it is lowest in peripheral blood smears, as parasitaemia in immune-competent individuals with VL is low. The reported sensitivity of direct microscopic examination of lymph node aspirates ranges from 52 to 58% (Zijlstra et al., 1992; Siddig et al., 1988), and for bone marrow aspirates from 52 to 85% (Zijlstra et al., 1992; Chowdhury et al., 1993; Bryceson, 1996).

Enlarged lymph nodes are typically observed in VL patients in Sudan, but are rare in patients from other countries. Spleen aspiration has been shown to be the most sensitive aspirate assay (93.1 to 98.7%) (Zijlstra et al., 1992). A safe procedure for splenic aspiration was developed in Kenya, but remains an invasive and complex technique (WHO, 1996; Bryceson, 1996). After the procedure the patient must be observed in the recumbent position for a minimum of 8 h in a facility where blood transfusion is available.

Splenic aspiration is not possible in non-cooperative children; it is difficult in those without a palpable spleen and is contra indicated in persons with active bleeding, thrombocytopenia, severe anaemia or jaundice, those in a moribund state, non-cooperative individuals and pregnant women. There is a small risk of fatal haemorrhage and several authors have reported iatrogenic morbidity and mortality (Boussery et al., 2001; Sundar et al., 2002).

One death was observed in a series of 671 splenic aspirates in Kenya (Kager et al., 1983; Siddig et al., 1988) and in a series of 3,000 in India (Thakur, 1997). Two episodes of fatal bleeding occurred following 9,612 splenic aspirates (0.02%) in a specialized treatment centre in India (Sundar and Rai, 2002). Thus, splenic aspirate is highly sensitive and specific, but can only be carried out under strictly controlled conditions, and is not suitable for use in first-line health centres.

**Culture method**

In culture method, the lesions and the adjacent normal-looking skin around it is cleaned, sterilized with 70% ethanol and allowed to dry. A small amount of the scraped tissue is inoculated in 10% of rabbit blood medium. The culture is then incubated at 25°C and examined for parasite growth by the inverted microscope and also light microscope every 4 days until promastigotes were seen or up to one month. After one month if nothing appears the sample is discarded as negative. The cultures are made at least in duplicates for each case on the liquid phase of Novy-McNeal-Nicolle (NNN) medium (Rahi et al., 2015).

Parasite culture is expensive and time-consuming, and requires expertise and expensive equipment. Its use is therefore restricted to referral hospitals or research centres.

**Isoenzyme analysis by electrophoresis**

In the last two decades, intrinsic characteristics, such as biochemical and molecular data were used for the classification of Leishmania isolates. Biochemical techniques, most notably isozyme electrophoresis (IE), provided an effective and reliable tool for characterization of Leishmania isolates (Rahi et al., 2015).

In this method, the parasite is submitted to isoenzyme electrophoresis on cellulose acetate plates. Each sample is mixed with a hypotonic enzyme stabilizer, maintained during 30 min on ice, centrifuged for 2 min at 3500 × g and then immediately run for electrophoresis.

Hitherto, it still represents the reference technique for Leishmania identification. The parasites can be identified by their enzymatic profile and grouped in taxonomic units termed zymodemes. However, isoenzyme analysis is time consuming and laborious, requires culturing and obtaining the profile of 10 to 20 different enzymes. Molecular methods, such as Southern hybridization and PCR-based methods have become available; that is, less laborious and more powerful to study variability between Leishmania species (Morales et al., 2001).

**Napier’s Aldehyde test**

In the Kala-azar endemic areas of India, Napier’s aldehyde test has been used for a long time. The test relies on the jellification caused by the binding of the serum globulins to the formaldehyde. The serum globulins increase in a variety of infections and hence, this test is rather non-specific. A positive reaction may also be seen in diseases like, Tuberculosis, Cirrhosis of liver and Malaria, etc. Furthermore, in kala-azar, the test becomes positive only when infection is at least three months old and may remain positive even after six months of cure. A range of assays were developed to detect anti-leishmanial antibody.

**Complement Fixation test**

The complete fixation test (CFT) is used to detect the presence of specific antibodies in the patient’s serum. This test is based on the use of complement, a biologically labile serum factor that causes immune cytolysis, that is, lysis of antibody coated cells. The first step is to heat the serum at 56°C to destroy patient’s complement. A measured amount of complement and antigen are then added to the serum. If there is presence of antibody in the serum, the complement is fixed due to the formation of Ag-Ab complex. If no antibody is present, then, the complement remains free. To determine whether the complement has been fixed, sheep
RBCs and antibodies against sheep RBCs are added. In the positive test, the available complement is fixed by Ag-Ab complex and no hemolysis of sheep RBCs occurs. As a result, the test is positive for the presence of antibodies. In the negative test, no Ag-Ab reaction occurs and the complement is free. This free complement binds to the complex of sheep RBC and its antibody to cause hemolysis, which results in the development of pink color.

**Direct Agglutination Test (DAT)**

In 1985, El Harith et al. (1986) developed a DAT for VL with high sensitivity and specificity and these values have been confirmed by other laboratories (Sinha and Sehgal, 1994; El-Safi and Evans, 1986). The test is semi-quantitative and uses microtitre plates with V-shaped wells in which increasing dilutions of serum or blood eluted from filter paper are mixed with stained killed *L. donovani* promastigotes. As the ongoing VL epidemic in Sudan (de Beer et al., 1991) created a pressing demand, the DAT was rapidly taken to the field.

Contradictory reports on its performance were soon published (Zijlstra et al., 1991; el Masum et al., 1995). A multi-centre study reported low reproducibility owing to problems reading the results and the heat- and shock sensitivity of the liquid antigen (Boelaert et al., 1999). A freeze-dried version of the test was developed to circumvent the latter problem, and this version showed similar diagnostic performance to the liquid version (Meredith et al., 1995; Abdallah et al., 2004; Jacquet et al., 2006).

In this method, culture of *L. donovani* promastigotes are freeze dried, trypsin-treated fixed and stained for making suspension. During infection with VL, circulating antibodies are produced against the surface antigens of the invading parasites. The DAT detects antibodies of *L. donovani* by direct agglutination in the blood or serum of infected patients. In the absence of human antibodies against Leishmania the DAT antigen accumulates at the bottom of the plate to form a dark blue spot indicating a negative result. If human antibodies against Leishmania are present then the antigen forms a pale blue film over the well, thus, indicating a positive result. DAT requires moderate technical expertise, reagents (for example, 2-beta Mercaptoethanol) and laboratory equipment, including calibrated pipettes and micro-titer plates etc (Emily et al., 2012). The test is performed at room temperature, while the antigens are stored under controlled temperature in freezer.

Since 1986, this test has been widely used for serodiagnosis of kala-azar. Studies showed sensitivity and specificity estimates of 94.8% (95% confidence intervals (CI), 92.7 to 96.4) and 97.1% (95% CI, 93.9 to 98.7), respectively (Chappuis et al., 2006).

The usefulness of the aforementioned serological tests is limited by their variable sensitivity or specificity, requirement of electricity, refrigeration, or a well equipped laboratory and high cost. Although highly sensitive and specific, the DAT requires substantial manipulation and can only be read after a minimum of 8 h incubation. Furthermore, despite very good accuracy, inter-observer discrepancy in routine DAT serology readings is common (Emily et al., 2012).

**Indirect Hemagglutination Test (IHA)**

This test is also based on the principle of antigen antibody reaction. The serum antibodies are conjugated with parasite antigens to observe agglutination. The BMA is collected from all patients by a posterior superior iliac spine puncture. Smears prepared from the aspirates are stained by Romanowsky stains, mostly May-Grünwald–Giemsa or Giemsa alone. On microscopic examination, amastigotes appeared as rounded or oval bodies, measuring 2 to 3 μm in length; these amastigotes are found to be extracellular or intracellular in the monocyte/macrophage. In some cases, a more sharply demarcated and characteristic ‘double spot’ appearance of Leishmania donovani bodies (LDB) are observed.

The fact that the detection by such assays depends on elevated levels of the specific immunoglobulin highlights the factor of sensitivity. Another complicating factor is the polyclonal and non-specific activation of B lymphocytes, a hallmark of VL infection, which produces large amounts of antibodies against irrelevant proteins and haptens. These vast amounts of activated B lymphocytes and the accompanying production of polyclonal antibodies have been advocated as the reasons for the increased false positivity in the IHA assay (Jamil et al., 2012).

**Immuno-fluorescent Antibody Test (IFAT)**

In this method, parasite antigen labeled with fluorescent dye is conjugated with serum antibodies and is observed under fluorescent microscope. This test has also been widely used. In IFAT, the antigen is prepared from promastigotes of Leishmania. Anti- *Leishmania* antibodies are detected using anti-IgG conjugated to fluorescein isothiocyanate. Samples are recorded as positive if promastigote cytoplasmatic or membrane fluorescence is observed at a serum dilution of 1: 40 or higher. The IFAT technique was traditionally considered a gold standard for the serological diagnosis of Leishmania infection with optimal performance measures with regards to sensitivity and specificity. This test is still considered by some authors to be a technical reference in diagnostic laboratory practices. However, its interpretation can be subjective depending on the operator’s skills and experience when interpreting results (Solano-Gallego et al., 2014).
Indirect immuno-no-fluorescent antibody (IFA) test

This test showed acceptable estimates for sensitivity (87 to 100%) and specificity (77 to 100%) (Sinha and Sehgal, 1994; Boelaart et al., 2004). This was developed using Leishmania promastigotes.

In Brazil, the IFA test was used by the Ministry of Health for testing dogs, which are presumed to be the most important reservoir for *L. chagasi*; but the need for a fluorescence microscope restricts their use to reference laboratories. Hitherto, only two antibody-detection tests were extensively evaluated for field use: the direct agglutination test (DAT) and the rK39 immunochromatographic test (ICT). Therefore in recent years, enzyme-linked immunosorbent assays (ELISAs) replaced the IFA test and DAT in humans.

Delayed-type hypersensitivity test

Delayed type hypersensitivity (DTH) or T-cell-mediated immunity is a group specific immune response. The Montenegro skin test (leishmanin skin test) is a test for DTH specific to leishmaniasis, but its role is limited. In this method, 0.5 ml of phenol-killed whole parasites (5 × 10^6 promastigotes) is injected on the volar aspect of the forearm of the patient. After 48 to 72 h, the size of induration is measured and compared with the size of induration produced by injection of a phenol-saline control in the other forearm. Presently, there is no available standardized leishmanin reagent. All leishmanins are said to be alike and non-specific. The test is negative in acute cases of VL due to the absence of DTH and is positive only in cases where kala-azar has been cured (Sundar and Rai, 2002).

Latex agglutination test

This test showed high specificity but equivocal sensitivity for the detection of leishmanial antigen in urine of patients with VL (Singh et al., 2009).

Enzyme Linked Immunosorbant Assay (ELISA)

This method utilizes soluble antigen or sonicated extract of promastigotes to capture antibodies specific to Leishmania. Though sensitive and specific, it may give cross reactions with infections like, malaria, tuberculosis and leprosy, etc at very low litres.

Dip-Stick Test

A test based on a 39-amino acid- repeat recombinant leishmanial antigen from *Leishmania chagasi* (rK39) was introduced into an enzyme-linked immunosorbent assay (ELISA) (Badaro et al., 1996; Zijlstra et al., 1998) and thereafter, an immune-chromatographic strip test 9Dip-Stick test) (Sundar et al., 1998). The latter is easy to use in the field and results made available after 15 min.

K39 is an epitope apparently conserved on amastigotes of Leishmania species that cause visceral infection; by use of laboratory ELISA testing, circulating anti-K39 and IgG is detectable in 95 to 100% of patients who have kala-azar, irrespective of geographic region (Mursalin et al., 2015). The initial study showed 100% sensitivity and 98% specificity (Sundar et al., 1998). rK39 was found to be more sensitive than DAT. An inverse correlation was found between anti-rK39 antibodies and the development of delayed-type hypersensitivity (DTH) responses among patients treated for VL.

Serological tests like the direct agglutination test (DAT), rK39 immunochromatographic tests and the Indirect Immunofluorescent Antibody test all have high sensitivity but cannot discriminate between past and current infections (Sundar and Rai, 2002; Sundar et al., 1998; Zijlstra et al., 2001).

Antigen-detection methods

Recently, Sarkari et al. (2002) described a urinary leishmanial antigen, a low-molecular-weight, heat-stable carbohydrate that was detected in the urine of VL patients. An agglutination test used to detect this antigen was evaluated in laboratory trials, using urine collected from well-defined cases and controls from endemic and non-endemic regions. This test showed 100% specificity and sensitivity between 64 and 100%, respectively (Attar et al., 2001). However, the sensitivity of this test was disappointingly low in clinically suspect patients in a VL-endemic area in Nepal (Rijal et al., 2004). Further work is ongoing, as this technique holds promise as a test of cure, for which none of the current serological tests is appropriate.

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 and in particular, in many developing countries (Schallig et al., 2001). However, in developing countries diagnosis is moved towards molecular identification by Southern Blotting and PCR.

This is in part due to 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 has 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).

**Southern blotting using DNA probes**

One of the first molecular characterization methods in use was Southern blotting using DNA probes. DNA probes for identifying Leishmania species 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) (Smith et al., 1989; Gramiccia et al., 1992; 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. This method is tedious as it requires cultivation of promastigotes, DNA extraction, gel electrophoresis, Southern blotting and hybridization (Van Eys et al., 1989).

**Random amplified polymorphic DNA (RAPD)**

This method 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 and McCleland, 1990). However, the RAPD method has one major drawback: it can only be used on cultured parasites free of contaminating the 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).

**Restriction fragment length polymorphism (RFLP)**

With RFLP a PCR amplicon was digested with a set of different restriction enzymes and resulting fragments separated according to molecular size using gel electrophoresis. For example, by digesting the amplified small subunit ribosomal RNA PCR product with the restriction enzyme RsaI, 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 sub-genus Viannia, namely *L. (V.) braziliensis*, *L. (V.) peruviana*, *L. (V.) guyanensis* and *L. (V.) lainsoni* (Victor et al., 1998).

Cupollo et al. (1995) exploited the variability of the transcribed non-coding regions between the small and large subunit rRNA genes to examine relationships in the Viannia sub-genus. In a method termed intergenic region typing (IRT), PCR amplification products were obtained for the rapidly evolving 1 to 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 intrain and inter-specific variation were observed and quantitative similarity comparisons used to associate different lineages. A complex evolutionary tree was obtained. Some species formed tight clusters (*L. equatoriensis*, *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. equatoriensis* and *L. lainsoni* clearly represent distinct lineages. Until now, no differences supporting the idea that *L. chagasi* and *L. infantum* are identical are found between *L. infantum* and *L. chagasi* (Mauricio et al., 2000).

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 sub-genus and the taxonomic status of a number of New World complexes and have partly been addressed by Cupollo 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 and Blackwell, 1999). This was 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, sequencing and mapping of Expressed Sequence Tags (ESTs) to PFG separated chromosomes and the generation of DNA sequence from the entire chromosomes.

**Polymerase chain reaction**

Molecular diagnosis exploiting PCR combines several advantages; it is minimally invasive, has a high sensitivity and specificity and capable of identifying relapses and re-infections in treated VL patients and can provide species identification (Minodier et al., 1997; Schonian et al., 2003).

Many different PCR assays targeted conserved and variable regions of kDNA minicircles (Noyes et al., 1998; Salotra et al., 2001; Adhya et al., 1995; Smyth et al., 1992; Maurya et al., 2005), genomic DNA, splice leader mini-exon (SLME) (Ramos et al., 1996), telomeric repeats (Chiurillo et al., 2001), rRNA gene (van Eys et al., 1992) and gpd63 (Dujardin et al., 2002) for the detection of parasites directly from human tissues. However, despite countless reports describing PCR in VL diagnosis, none of these assays are used as a diagnostic tool in clinical setting.

Diagnosis by polymerase chain reaction (PCR) over the years shows 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 aspirate (BMA) and lymph node aspirates (LNA) 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.

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 individuals 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).

In post-kala-azar dermal leishmaniasis (PKDL), PCR 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). 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., 1998b) 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). Until now, only one report was published on the use of realtime 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 so far, has hardly been addressed is the standardization of PCR technology for the
diagnosis of Leishmaniasis. Most laboratories use ‘in house’ PCR methods based on different primer pairs and DNA targets (Lachaud et al., 2002).

**Nucleic acid sequence-based amplification**

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 and 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). NASBA can be used for the accurate quantification of RNA levels, which allows the accurate quantification (that is, 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 fills the niche where NASBA could be of value.

**DNA microarrays**

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 upregulated in expression in amastigotes that were identified. These are now being tested as new vaccine candidates; some cocktails of them appear to be effective as DNA vaccines in mice.

**CONCLUSION**

Currently, the molecular diagnosis of VL solely relies on PCR based assays of various throughputs as extensively reviewed by Reithinger and Dujardin (2007). Recently, chromatography based Oligo-C test and NASBA have been introduced (Deborggraeve et al., 2008) and their sensitivity and specificity determined in Sudan (Saad et al., 2010), East Africa (Mugasa et al., 2010) and Kenya (Basiye et al., 2010) in clinical samples from VL patients.

In the clinical setting, if there is a negative PCR result and a high level of clinical suspicion, then other methods of detection such as splenic aspiration or serology could be used (Sundar et al., 2006). The sensitivity and specificity of any PCR assay depends on many factors and precautions must be taken at each and every step starting from sample collection till DNA isolation. Strict measures like complete separation of pre PCR and post PCR regions and negative controls with each set of experiment will help in achieving the high specificity level.

A basic molecular biology laboratory with minimum infrastructure; a thermocycler and UV transilluminator and gel electrophoresis unit are a onetime investment and if they can be provided, PCR could be performed in an endemic setting for routine diagnosis. 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 it is still a major problem, for example, 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 Leishmania disease and in particular for HIV/Leishmania coinfections.

**REFERENCES**


