Methods for the detection of blood parasites

A. H. MOODY
Department of Clinical Parasitology, Hospital for Tropical Diseases,
London, UK

P. L. CHIODINI

Introduction

The diagnosis and identification of blood parasites is becoming ever more important with the increase in imported parasitic disease in the temperate zone. Any clinical haematology laboratory can expect to be called upon to diagnose these parasites, especially malaria, and a high level of competence is required. This review considers the imported blood parasites that could be encountered and discusses the merits of the various diagnostic tests available.

Protozoa

Malaria

The accepted laboratory procedure for the diagnosis of malaria is the preparation and examination of Giemsa or Field’s stained blood smears under the light microscope (Warhurst & Williams 1996; General Haematology Workforce 1997). Smears may be prepared directly from a finger prick blood sample. Venipuncture specimens collected in EDTA coated tubes are also acceptable, but must be examined promptly to prevent alteration of parasite morphology due to the sequestrine effect. Both thick and thin smears should be made. The thick smear, which increases by a factor of 20–30 the number of red blood cells per given area on the slide compared to the thin film, is much more sensitive than the thin film for detection of a malarial infection. The thin smear is superior to the thick for speciation.

This technique has undergone very little improvement over the last 100 years. Although considered the gold standard, diagnosis of malaria using blood films can be problematic: it requires up to 60 min of preparation time, is labour intensive and interpretation of the result requires considerable expertise, particularly at low levels of parasitaemia Warhurst & Williams (1996). In addition, in patients with Plasmodium falciparum malaria, the parasites are sequestered in tissue capillaries for part of the asexual cycle and, in a synchronous infection with this species, may not always be present in the peripheral blood. Thus a P. falciparum infection might be missed unless blood samples are repeated daily, or more frequently if the clinical picture dictates. Furthermore, detection of parasites by blood film on follow-up of treatment does not assess their viability (Srinavasan et al. 2000).

Assessment of parasitaemia by examination of blood smears

Warhurst & Williams (1996) discuss several methods employed to quantify malaria parasites in thick blood smears. One technique is to count the total number of parasites per 200 white blood cells (WBC) and multiply this number by 40 to give the number of parasites/µl, assuming that there are 8000 WBC/µl blood. A second method involves making a thick smear with a known volume of blood (5 µl), and staining with Giemsa stain before counting all the parasites on the smear. The total parasite count is divided by 5 to obtain the number of parasites/µl. A similar technique again uses a defined amount of whole blood to make a thick smear and then the parasites present in 100 high-power fields (HPF) are counted. One parasite/100 HPF is assumed to be the equivalent to 50 parasites/µl. A thick smear is generally considered negative if no parasites are seen after 10 min searching. Parasite levels may also be quantified by examination of a thin blood smear. This is usually accomplished by counting the number of parasites/1000 red blood cells (RBC) and expressing as a percentage the number of erythrocytes infected, or again, by counting the number of parasites per 200 WBC as described for the thick film.
A weakness in the estimation of parasite levels by counting parasites against a particular number of WBC is the assumption that all blood samples contain 8000 WBC/μl. If a suitable instrument is available, it is much better to count the WBC/μl of each blood sample under investigation, and so obtain an accurate conversion factor for each sample. For example, if there are 10 parasites/200 WBC and the total WBC is 10,000/μl then there are 500 parasites [i.e. (10,000/200) × 10] parasites/μl.

The major disadvantage of the thick smear is that it is difficult to read and the necessary expertise may be unavailable. Using one method to evaluate the intensity of the infection, the examiner should, in theory, only count thick-smear fields containing 20 WBC/HPF. In reality, fields containing 20 WBC are often too thick to count accurately, whereas the fields that are easiest to read are those that contain only five or six WBC each. It has been suggested that the microscopist should be able to read print through a well-prepared, thick blood smear before it is stained. Warhurst & Williams (1996) report that examination of thin blood smears is only one tenth as sensitive as examination of thick blood smears for the quantification of malarial parasites. Although identification of the Plasmodium species present is much easier using thin smears. Most laboratories involved in the identification and quantification of malaria parasites by microscopy therefore produce both thick and thin blood smears. In conclusion, even though examination of blood smears is accepted as the current, universal ‘gold standard’ for malaria diagnosis, no single method for estimation of parasitaemia is used by all investigators, which can lead to difficulties in comparing data between different studies (Hanscheid & Valadas 1999).

Fluorescent microscopy

Three techniques using fluorescence of the parasite for the diagnosis of malaria have been described. The quantitative buffy coat or QBC11® method (Baird et al. 1992; Benito et al. 1994; Clendeman, Long & Baird 1995) which is available as a commercial kit (Becton Dickinson, Franklin Lakes, NJ, USA); the Kawamoto acridine orange process (Kawamoto 1991a; Kawamoto 1991b; Kong & Chung 1995; Bosch et al. 1996; Gay et al. 1996; Lowe et al. 1996) and the use of benzothiocarboxyypurine (BCP) (Makler et al. 1991; Cooke et al. 1992). These three techniques are rapid and relatively easy to perform (when there are > 100 parasites/μl) and demonstrate sensitivity and specificity equivalent to those achievable by examination of stained thick smears.

Both the QBC and Kawamoto methods use acridine orange (AO) as the fluorochrome to stain the nucleic acids of any malaria parasites in the sample. BCP is another fluorochrome which stains nucleic acids. Although AO is a very intense fluorescent stain, it is nonspecific and stains nucleic acids from all cell types. Consequently, the microscopist using AO has to learn to distinguish fluorescent-stained parasites from other cells and cellular debris containing nucleic acids. The sensitivity of AO staining at parasite levels of < 100 parasites/μl has been reported to range from 41.1% to 93% (Delacollet & VanderStuyft 1994). The specificity of AO staining for P. vivax infections was reported to be about 52%, whereas that for P. falciparum infections was around 93% (Hakim et al. 1993). The BCP method has a reported sensitivity and specificity of > 90% (Cooke et al. 1993). An important limitation of methods based on AO and BCP is their inability to differentiate easily between Plasmodium spp. In addition, AO is considered hazardous and has special disposal requirements.

Comparing methodologies, both the QBC and the BCP fluorescent methods are more demanding technically than the Kawamoto AO method (Hakim et al. 1993; Hind et al. 1994) and require special equipment and supplies. The QBC method requires a particular centrifuge and haematocrit tubes and was reported to increase costs to about US$ 1.70/sample (Craig & Sharp 1997). The BCP method also requires a good fluorescent microscope, with a high intensity mercury vapour or quartz halogen lamp. The simpler Kawamoto method employs a pair of fluorescence filters and readily available AO and can make use of the sunlight as the exciting light source (if a shield to surround the observer’s eyes is used). If sunlight is not used this technique also requires a high-intensity halogen lamp.

In spite of its limitations, including the requirements for special training, expensive equipment and supplies, fluorescence microscopy for the rapid detection of malaria parasites in blood is a viable alternative to examination of Giemsa stained smears.

Detecting specific nucleic-acid sequences

Another approach to the laboratory diagnosis of malaria is based on the detection of nucleic acid sequences specific to Plasmodium. Several PCR assays have been developed for the diagnosis of malaria. The 18S rRNA gene has been used as a target for the differentiation of Plasmodium species by nested PCR and reverse transcription-PCR (Barker et al. 1992; Snounou et al. 1993). Other DNA targets such as the circumsporozoite protein gene have also been
investigated for species specific regions. The large-subunit rRNA gene is extensively conserved within *Plasmodium* species and is suitable as a genus-specific target region; the amplified target sequence is detected by internal probes or analysed by gel electrophoresis (Kain *et al.* 1993). The major advantage of using a PCR-based technique is the ability to detect infection in patients with low parasitaemia: infection with five parasites/µl can be detected with 100% specificity (Kawamoto *et al.* 1996). PCR-based techniques have been used to screen blood donors in Ho Chi Minh City, Vietnam, an area where malaria is endemic.

The sensitivity of PCR-based methods is greater than microscopy and, following treatment, PCR yields positive results longer than microscopic examination (Kain *et al.* 1994). Several workers have investigated the persistence of parasite DNA in peripheral blood following antimalarial treatment. In one report, PCR remained positive for a median of 144 h compared to 66 h for microscopy (Sethabutr *et al.* 1992; Seesod *et al.* 1993). Such microscopically negative but PCR positive specimens probably had either subpatent parasitaemia or circulating *P. falciparum* DNA. Kain *et al.* (1994) also reported that if PCR yields positive results for 5–8 days after treatment, therapeutic failure, possibly due to drug resistance, might be predicted.

The variable PCR results in different studies (Table 1) reflect a number of variations in PCR technique from collection and storage of the specimens, DNA extraction, selection of primers, amplification conditions, and analysis of amplified product. Further advances in PCR technology may soon allow viable parasites to be distinguished from nonviable and facilitate use of PCR-based procedures in the field (Wataya *et al.* 1993). Although PCR-based methods are arguably the most sensitive method for detection of patent malarial infections, they are also particularly useful for studies on strain differences, mutations, and genes involved in drug resistance in the parasites and to show the relationship between strains associated with different outbreaks of malaria. Use of PCR-based technology for malaria diagnosis may therefore become clinically relevant for acute diagnosis in both field and reference laboratory situations.

**Antigen detection**

Although alternative methods to stained blood films for diagnosis of malaria have been around for a considerable time (Taylor & Voller 1993), the original fluorescent antibody detection tests for malaria were limited in sensitivity and in their ability to distinguish active from prior infections. The new generation antigen capture tests are capable of detecting fewer parasites and of producing a more rapid result (10–15 min). They are commercially available as kits, which include all the necessary reagents, and do not require extensive training or equipment to perform or to interpret their results.

Two parasite antigens are currently used in the new, rapid immunochromatographic tests: Histidine-rich protein-2 (HRP-2) (Rock *et al.* 1987), a water-soluble protein which is expressed by *P. falciparum* and parasite lactate-dehydrogenase (pLDH) antigen (Makler *et al.* 1998), present as separate isomers for all four *Plasmodium* species infecting humans. Both of these antigens are also produced by gametocytes (Odoula *et al.* 1987; Kilian *et al.* 1997). The latest antigen capture tests are rapid and simple to perform, and have detection limits comparable to those of high-quality microscopy (i.e. 100–200 parasites/µl).

**HRP-2 immunochromatography-based assays**

At parasitaemias of 60–100 parasites/µl, the HRP-2 based tests are > 90% sensitive and > 90% specific for *P. falciparum*, compared with thick-smear microscopy.

<table>
<thead>
<tr>
<th>Table 1. PCR-based detection of <em>Plasmodium</em> DNA in blood samples (Modified from Weiss 1995)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmodium</strong> sp.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td><em>P. falciparum/P. vivax</em></td>
</tr>
<tr>
<td><em>P. vivax</em></td>
</tr>
<tr>
<td><em>P. falciparum/P. vivax</em></td>
</tr>
<tr>
<td><em>P. malariae/P. ovale</em></td>
</tr>
</tbody>
</table>

Currently, two such tests are commercially available: the ParaSight® F-test (Becton Dickinson) and the ICT Malaria Pf Test® (Amrad ICT Diagnostics, Sydney, Australia).

Both tests can be performed on finger-prick blood samples. They deploy labelled monoclonal antibodies to HRP-2 in a mobile phase, to capture HRP-2 antigen from *P. falciparum* and a further stripe of anti HRP-2 antibody immobilized onto nitro-cellulose strips, to capture labelled antigens in a dipstick format. In each test, a positive result is indicated by the appearance of a red line on the test strip at the site where the monoclonal antibodies are immobilized. Both tests also contain a built-in control line, which must appear for the test to be considered valid.

A comparison of the ParaSight® F antigen-capture test with PCR-based diagnosis (Peyron et al. 1994) gave a reported sensitivity and specificity of the ParaSight® F-test of 80% and 97%, respectively, compared with the results of PCR.

Other studies on the sensitivity and specificity of the ParaSight® F-test for the detection of *Plasmodium falciparum* in blood samples. (Makler & Hinrichs 1993; Schiff et al. 1993; Beadle et al. 1994; Miller et al. 1994; Peyron et al. 1994; Premji et al. 1994; Dietz et al. 1995; Verle et al. 1996; Kodisinghe et al. 1997; Singh et al. 1997) have shown an average sensitivity of 77–98% and specificity of 83–99%.

Although HRP-2-based immunochromatographic tests permit rapid diagnosis of *P. falciparum* malaria, they do have limitations in their use. Firstly, since HRP-2 is only expressed by *P. falciparum*, tests based on the detection of HRP-2 give negative results for samples containing *P. vivax*, *P. ovale* or *P. malariae*. Thus, cases of nonfalciparum malaria may be misdiagnosed as malaria-negative if the only diagnostic test used is based upon HRP-2 detection. Another limitation is the persistence in the blood of HRP-2 after the clinical symptoms of malaria have disappeared and the parasites have apparently been cleared from the host. Results of investigations with the ParaSight® F-test on blood taken 0–14 days after treatment of falciparum malaria with artemether showed persistence of HRP-2 in 16 of 75 blood samples at day 14 after treatment had finished.

Tests for HRP-2 may therefore be difficult to interpret when screening indigenous people of malaria-endemic areas, who may have persistent, low-level parasitaemia, yet not be ill (Verle et al. 1996). The reason for the persistence of the HRP-2 antigens is not well understood; it may reflect the presence of latent, viable parasites (possibly the result of treatment failure) or of soluble, antigen/antibody complexes (Humar et al. 1997). The persistence may also depend upon the type of antimalarial therapy instituted. Schiff et al. (1993) noted that 10% of patients treated with Fansidar® had detectable HRP-2 antigen on day 14. Using the ParaSight® F-test to investigate 19 patients given unspecified antimalarial chemotherapy, Humar et al. (1997) detected HRP-2 antigen in 68% of the patients on day 7 after initiation of treatment and in 27% on day 28. The HRP-2 signal has been shown to persist for 19 days after apparently effective quinine therapy. Karbwang et al. (1996) also detected persistent HRP-2 antigen during and after artemether therapy, acknowledging that the HRP-2 signal was of no value during the first week of treatment but appeared to be a precise indicator of treatment failure under field conditions, when it was detected on day 14 post-treatment. More studies are required in this area.

Other limitations are specifically related to technical aspects of the test system. For example, the monoclonal IgG antibody utilized in the ParaSight® F may cross-react with serum rheumatoid factor and be the cause of a false-positive response (Laferi et al. 1997).

**pLDH-based immunochromatographic assays**

Parasite lactate dehydrogenase (pLDH) (Makler et al. 1993; Piper et al. 1996) is a glycolytic enzyme present in all species of malaria parasites of humans. Furthermore, pLDH from *P. falciparum* can be assayed in the presence of host LDH utilizing the coenzyme 3-acetylpyridine adenine dinucleotide (APAD), an analogue of nicotinamide adenine dinucleotide (NAD) (Piper et al. 1999). Although the Michaelis–Menten constants (k_m) are similar, the k_cat (turnover number) of pLDH in the presence of APAD is much greater than that of the human enzyme with the same coenzyme (Gomez et al. 1997). This characteristic is due in part to conformational changes that occur in the enzyme (Dunn et al. 1996).

pLDH-based assays are available in two formats: a semi-quantitative, dry dipstick (OptiMAL®, Flow Inc., Portland, OR, USA) and a 96 well, quantitative, immunoenzymatic capture, plate assay (Piper et al. 1999). The latter technique captures pLDH on a microtitre plate with a specific anti pLDH monoclonal antibody, then quantifies the amount of enzyme captured with an enzyme reaction utilizing APAD. The bound pLDH activity correlates well with viable parasite density on initial diagnosis. But the pLDH activity has also been shown to parallel the level of viable parasites during therapy (Makler & Heinrichs 1993; Gomez et al. 1997; Piper et al. 1999). The latter observation is especially important, since this
assay could thus be used to follow patient progress during antimalarial therapy, and alert the physician to the presence of a drug-resistant infection if pLDH levels fail to decline in sequential samples.

Low levels of pLDH cannot easily be measured colorimetrically by this enzymatic method because of the presence of nonspecific reductases in blood lysates. Because of this, two assays have been formatted that utilize monoclonal antibodies (mAbs) which capture the pLDH. These mAbs were raised against pLDH purified from *P. falciparum* infected erythrocytes. They vary in respect of their reactivity with pLDH isoforms from the different human malarial species. Two of the mAbs are pan-specific, recognizing all four species of malaria. A third mAb recognizes only *P. falciparum* LDH isoforms. The monoclonal antibodies utilized in the OptiMAL test have been exhaustively tested for cross-reactivity with LDH from *Leishmania*, *Babesia* and pathogenic bacteria or fungi and no evidence of such cross-reactivity has been found (Makler & Heinrichs 1993).

The immunochromatographic strip test (OptiMAL) which is based on these mAbs uses a gold-conjugated pan-specific antibody (6C9) to capture pLDH from all species of *Plasmodium* from humans. The blood/antibody complex travels along a nitro-cellulose strip on which are two further monoclonal antibody capture stripes, one specific for *P. falciparum* LDH isoforms and a second pan-specific monoclonal antibody (19G7) for capturing all four *Plasmodium* species. A goat antimouse monoclonal antibody capture control line is present to indicate a successfully performed test. The gold conjugated antigen/antibody complex builds as a red line at the capture stripe. The presence of three lines (control + pan-specific + Pf specific) indicates *P. falciparum* antigen captured. Two lines (control + pan-specific but not Pf specific) indicates a species other than *P. falciparum*.

Trials of the OptiMAL test have been undertaken in many centres (John et al. 1998; Palmer et al. 1998; Quitana et al. 1998; Hunt Cooke et al. 1999; Moody et al. 2000) and have reported sensitivities of 92–98%, with a specificity of 94%, for *P. falciparum* and a sensitivity of 96% for *P. vivax*.

Studies to evaluate pLDH levels in sequential blood samples taken from patients undergoing antimalarial chemotherapy demonstrated that pLDH levels followed peripheral parasitaemia and fell to undetectable values 3–5 days after quinine therapy, which could provide a valuable tool in monitoring antimalarial therapy (Srinivasan et al. 2000). Clearance of parasitaemia and clearance of pLDH appear to parallel each other.

In the past few years, efforts to replace the traditional and tedious reading of blood smears have led to techniques for the detection of malaria parasites with a sensitivity equivalent to or better than microscopy (Taylor & Voller 1993). Immunochromatographic dipsticks offer the possibility of a more rapid, nonmicroscopic method for malaria diagnosis, thereby saving on training and time.

**Babesiosis**

Babesiosis is caused by haemoprotozoan parasites of the genus *Babesia*. While more than 100 species have been reported, only a few have been shown to cause human infection. *Babesia microti* and *Babesia divergens* have been identified in most human cases (Hoare 1980), but variants (considered different species) have been seen in malaria-endemic areas where *Babesia* can easily be misdiagnosed as *Plasmodium*.

In the north-eastern USA, the blacklegged deer tick (*Ixodes scalpularis*) is the principal vector transmitting the aetiologic agent, *Babesia microti*. This tick is also the primary vector of the Lyme disease spirochaete, *Borrelia burgdorferi* (Benach & Habicht 1981). In areas where these diseases are endemic, concurrent infections by both pathogens in *P. leucopus* and in *I. scapularis* are common, but far fewer cases of human babesiosis have been reported than Lyme disease. Most *Babesia microti* infections are probably asymptomatic (Ruebush et al. 1977), as indicated by serological surveys. Manifestations of disease include fever, chills, sweating, myalgias, fatigue, hepato-splenomegaly and haemolytic anaemia. Symptoms typically occur after an incubation period of 1–4 weeks, and can last several weeks. The disease is more severe in patients who are immunosuppressed, splenectomized, and/or elderly. Previous splenectomy is the major risk factor for human infection with *B. divergens* and cases tend to be more severe (frequently fatal) than those due to *B. microti*, where clinical recovery usually occurs.

**Diagnosis.** *Babesia microti* and *Babesia divergens* are intraerythrocytic parasites and examination of Giemsa-stained blood smears is considered the most useful diagnostic procedure. The tetrad (Maltese cross) forms of *Babesia microti* are a primary diagnostic characteristic for infection with this species. However, the predominant form in most blood smears closely resembles ring stages of *Plasmodium* spp. (Garnham 1980), with small to large cytoplasmic vacuoles. Therefore, it is sometimes difficult to differentiate *Babesia microti* from *Plasmodium* spp., especially early ring stages of *Plasmodium falciparum*. 

Unlike *Plasmodium*, *Babesia* does not form pigment. *Babesia divergens* forms characteristic widely divergent pyriform pairs at the periphery of the erythrocyte and this species of *Babesia* should be less easily confused with malaria parasites.

*Babesia* infections in humans trigger humoral immune responses. An indirect immunofluorescent antibody (IFA) assay can be used for the diagnosis of clinical cases. Although high titres have been detected in patients during the acute phase, a cut-off point of 1:64 is generally accepted as diagnostic in IFA testing (Ruebush *et al.* 1977). The *B. microti* IFAT has a reported sensitivity of 88–96% and specificity of 90–100%.

Sub-inoculation of blood samples from patients into hamsters may provide a diagnosis by amplifying the parasitaemia. In addition, the use of the polymerase chain reaction (PCR) has proven to be useful in the diagnosis of zoonotic *Babesia* infections. By using genus- and species-specific primers, a definitive diagnosis can be made within a day. In areas endemic for *Babesia microti*, the infection rates in mouse populations can reach as high as 60%.

**Ehrlichiosis**

During the past 10 years, two tick-borne diseases caused by *Ehrlichia* spp. have been recognized in the United States. Human Monocytic Ehrlichiosis (HME) was first described in 1986. It is caused by *E. chaffeensis*, which was only identified in 1991. Human Granulocytic Ehrlichiosis (HGE), an alternative form of HME, was recognized as a new disease in 1993. Its causative agent is still uncertain, though it is similar to *Ehrlichia equi* described from horses.

Ehrlichiosis is characterized by fever associated with the presence of clusters of small organisms seen in circulating monocytes on Giemsa staining. Serology by IFA using *E. chaffeensis* infected cells is the preferred method of diagnosis (Taylor *et al.* 1998).

**Trypanosomiasis**

African trypanosomes are protozoan haemoflagellates belonging to the *Trypanosoma brucei* complex. Two morphologically indistinguishable subspecies cause distinct disease patterns in humans; *T. b. gambiense* causes West African sleeping sickness and *T. b. rhodesiense* causes East African sleeping sickness. A third member of the complex, *T. b. brucei*, does not infect humans under normal conditions.

**Life cycle.** *T. brucei* is transmitted by tsetse flies of the genus *Glossina*. The fly ingests parasites when it takes a blood meal from an infected mammal. The parasites multiply in the fly, going through several developmental stages in the insect gut and salivary glands (procyclic trypanosomes, epimastigotes, and metacyclic trypanosomes). The cycle in the fly takes approximately 3 weeks. When the fly bites another mammal, metacyclic trypanosomes are inoculated, and multiply in the host’s blood and extracellular fluids such as cerebro-spinal fluid (Omerod 1979). Humans are the main reservoir for *T. b. gambiense*, whilst wild game animals are the main reservoir for *T. b. rhodesiense*.

*T. b. gambiense* is found in foci in large areas of West and Central Africa. The distribution of *T. b. rhodesiense* is much more limited, being found in East and South-east Africa.

**Clinical features.** Infection occurs in three stages. A trypanosomal chancre can develop at the site of inoculation. This is followed by a haemolympathic stage with symptoms that include fever, lymphadenopathy, and pruritus. In the meningoencephalitic stage, invasion of the central nervous system can cause headaches, somnolence, abnormal behaviour and lead to coma. The course of infection is much more acute with *T. b. rhodesiense* than with *T. b. gambiense*.

**Laboratory Diagnosis.** The diagnosis rests upon demonstrating trypanosomes by microscopic examination of chancre fluid, lymph node aspirates, blood, bone marrow, or, in the late stages of infection, cerebrospinal fluid. A wet preparation of blood or where indicated CSF should be examined for motile trypanosomes in addition to a fixed smear stained with Giemsa (or Field’s) stain. Concentration techniques can be used prior to microscopic examination. For blood samples, these include Giemsa staining of thick blood smears, examination of Giemsa stained buffy coat preparations from haematocrit tubes (Woo & Hawkins 1975) or use of the quantitative buffy coat (QBC®) technique. Mini anion-exchange columns, which use the difference in electrical charge on the surface of the trypanosomes from that of the blood cells to effect a separation on an anion-exchange chromatography column (Lumsden *et al.* 1979) detects as few as 5–10 trypomastigotes per millilitre of blood. For other samples such as cerebrospinal fluid, concentration techniques include centrifugation followed by examination of the sediment. Isolation of the parasite by inoculation of rats
or mice is a sensitive method, but its use is limited to T. b. rhodesiense.

The card indirect agglutination test for trypanosomiasis (CIATT) is a rapid, simple test, which is based on the detection of trypanosome antigens. The test has undergone evaluation in a multi centre study carried out in Ghana, Côte d’Ivoire, Tanzania and Uganda (Penchienier et al. 1991; Enyaru et al. 1998; Akol et al. 1999). Results from the CIATT agree well (60–90% concordance) with results from PCR tests.

**American trypanosomiasis**

**Life cycle.** The protozoan parasite, *Trypanosoma cruzi*, causes Chagas’ disease, a zoonotic disease that can be transmitted to humans by blood-sucking reduviid bugs. The reduviid bugs become infected by feeding on human or animal blood that contains circulating parasites. The parasites multiply and differentiate in the insect gut, yielding infective metacyclic trypomastigotes. During a subsequent blood meal on a second vertebrate host, trypomastigotes are released in the insect faeces near the site of the bite wound. The host becomes infected through breaks in the skin, mucous membranes or conjunctivae. Inside the host, the trypomastigotes circulate in the peripheral blood and eventually invade the host cells, particularly striated muscle and gut tissue, where they differentiate into intracellular amastigotes. The amastigotes multiply and differentiate into trypomastigotes, which are released into the circulation as blood stream trypomastigotes. The blood stream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector.

*T. cruzi* can also be transmitted through blood transfusion, transplacentally, and in laboratory accidents.

Chronic Chagas’ disease is a major health problem in many Latin American countries. With increased population movements, the possibility of transmission by blood transfusion has become more substantial, for example in the United States.

**Clinical features.** A local lesion (Chagoma) can appear at the site of inoculation. The acute phase is usually asymptomatic, but can present with manifestations that include fever, anorexia, lymphadenopathy, mild hepatosplenomegaly, and myocarditis. Most acute cases resolve over a period of 2–3 months into an asymptomatic chronic stage. The symptomatic chronic stage may not occur for years or even decades after initial infection. Its manifestations include cardiomyopathy (the most serious manifestation), megacolon and megaesophagus.

**Laboratory Diagnosis.** Demonstration of the parasite is the diagnostic procedure of choice in acute Chagas’ disease. Trypanosomes may be seen on microscopic examination of fresh anticoagulated blood, or fresh buffy coat for motile parasites, which are seen only in the early acute stage of the infection. Examination of thin and thick blood smears stained with Giemsa stain may reveal trypomastigote stage organisms, characterized by their typical ‘c’ shape and large kinetoplast.

Isolation of the agent may be undertaken by: (a) inoculation into mice (b) culture in specialized media (e.g. NNN medium). Xenodiagnosis, where uninfected reduviid bugs are fed on the patient’s blood and the bug’s gut contents examined for parasites 4 weeks later, is occasionally used.

Serology is the diagnostic method usually deployed for the chronic phase of the infection when the trypomastigotes have virtually disappeared from the peripheral blood. ELISA, IFAT and several agglutination methods are available.

Recent studies have focused on the sero prevalence of *T. cruzi* in blood donors at risk from infection, an area where more work is needed (Leiby et al. 1999).

**Leishmaniasis**

Leishman and Donovan (Leishman 1903) first described the parasite causing visceral leishmaniasis in 1903, each separately demonstrating parasites in stained smears from the spleen of patients suffering from a malaria-like illness. This became known as visceral leishmaniasis and its causative agent was named *Leishmania donovani*. In 1908 Nicolle reported that mammals including dogs could act as reservoir hosts for *Leishmania*. In 1927, Adler & Theodor using human volunteers, proved that the leishmania parasite could be transmitted by phlebotomine sandflies.

Trypanosomatid parasites of the genus *Leishmania* give rise to a variety of disease manifestations, collectively known as leishmaniasis. Leishmaniasis is prevalent throughout the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe (Old World) and South and Central America (New World). It is estimated that approximately 12 million people are currently infected and a further 367 million in 88 countries are at risk of acquiring leishmaniasis, 72 are developing countries, 13 of them are among the least developed in the
The annual incidence rate is estimated to be 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis.

**Visceral leishmaniasis or Kala-azar (Hindi: **kala = black, azar = sickness).** The aetiological agents belong to the *Leishmania donovani* complex, *L. d. donovani* and *L. d. infantum* in the Old World and *L. d. chagasi* in the New World. The Old World species are transmitted by the sandfly vector *Phlebotomus*. Humans, wild animals and domestic animals are known to act as reservoir hosts. *Lutzomyia longipalpis* is the only sandfly vector that has been implicated in the transmission of the New World species of *Leishmania* and wild and domesticated dogs are known to serve as reservoir hosts. Visceral leishmaniasis is endemic in the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe, South and Central America. The distribution of VL in these areas however, is not uniform; it is patchy and often associated with areas of drought, famine and densely populated villages with little or no sanitation. In endemic areas children below the age of 15 are commonly affected. In sporadic and epidemic cases of VL, people of all ages are susceptible with males at least twice as likely to contract the disease than females, except those who have conferred immunity due to past infection. Ninety percent of VL cases occur in Bangladesh, India, Nepal and Sudan. In the Mediterranean basin 1.5–9% of AIDS patients develop visceral leishmaniasis and 25–70% of the adult VL cases are related to HIV infection. Both the New World and the Old World forms of the disease display similar symptoms and are often complicated by secondary infections.

**The Leishmania life cycle.** The sandfly vector becomes infected when feeding on the blood of an infected individual or an animal reservoir host. The leishmania parasites live in the macrophages as round, nonmotile amastigotes (3–7 micrometers in diameter). During the blood-meal, the sandfly ingests the macrophages and the amastigotes are released into the stomach of the insect. Most immediately the amastigotes transform into the motile, elongated (10–20 μm) flagellate promastigote form. The promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission. Four to five days after feeding the promastigotes move forward to the oesophagus and salivary glands of the insect. When the sandfly next feeds on a mammalian host, its proboscis pierces the skin, saliva containing anticoagulant is injected into the wound to prevent the blood from clotting and the leishmania promastigotes are transferred to the host along with the saliva. Once in the host the promastigotes are taken up by macrophages where they rapidly revert to the amastigote form. The leishmania are able to resist the microbicidal action of the acid hydrolases released from the lysozymes and so survive and multiply inside the macrophages, eventually leading to their lysis. The released amastigotes are taken up by other macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow. The term Kala-azar, used to describe established VL, originally referred to Indian VL with its characteristic blackening or darkening of the skin of the hands, feet, face and abdomen (Lainson & Shaw 1987).

Visceral leishmaniasis can be complicated by serious secondary bacterial infections such as pneumonia, dysentery and pulmonary tuberculosis, which often contribute to the high fatality rate of VL patients. Other, rarer complications include haemolytic anaemia, acute renal damage and severe mucosal haemorrhage.

Preliminary diagnosis is based on the symptoms and clinical signs of visceral leishmaniasis such as splenomegaly, hepatomegaly and high undulating fever. However, these alone cannot differentiate VL from other similar conditions such as malaria, relapsing fever, liver abscess and trypanosomiasis.

**Parasitological diagnosis**

**Splenial aspirate or liver biopsy.** Looking for parasites in the spleen and liver is one of the most accurate methods available to determine *Leishmania* infections. Splenic aspirate is preferred to liver biopsy.

Ninety percent of active cases show parasites in splenial aspirates. Part of the splenic aspirate can be used to make smears for direct microscopic examination and the rest should be cultured. *L. donovani* grows well on Novy–MacNeal–Nicolle (NNN) or Schneider’s insect medium supplemented 10% v/v foetal calf serum, although other suitable growth media can be used just as well. Liver biopsy material is less likely to demonstrate parasites on direct examination or on culture, but histological examination may show amastigotes in Kupffer cells in the portal system.

**Bone Marrow Aspirate.** Bone marrow aspiration is a safer method than splenic aspirate or liver biopsy. It is less likely to demonstrate parasites in stained films (Williams 1995), but on culture it can give positive results in up to 80% of cases.

Lymph node puncture gives positive results in 60% of cases where lymph nodes are enlarged. Juice is extracted from any enlarged lymph gland and subjected to both direct examination and to culture to give the best chance of diagnosis.

**Buffy coat.** Finding *Leishmania* parasites in blood is sometimes possible in patients with Kala-azar from India. Blood in anticoagulant is centrifuged at 2000 g for 10 min and the cells from the buffy coat removed and used to prepare smears and inoculate cultures. Amastigotes can be found in and around macrophages. The volume used for inoculation of cultures is important: addition of 1–3 drops to NNN or Schneider’s medium has given successful results (Lopez-Velez *et al.* 1995; Williams 1999).

**Serological diagnosis.** The IFAT is one of the most sensitive tests available and is based on detecting antibodies, which are demonstrable in the very early stages of infection and undetectable 6 to 9 months after cure. If the antibodies persist in low titres it is an indication of a probable relapse. Titres above 1/20 are significant and above 1/128 are diagnostic (Williams 1995). There is a possibility of a cross-reaction with antityranosomal antibodies. However, this can be overcome by using *Leishmania* amastigotes instead of promastigotes as the antigen (Gari-Toussaint *et al.* 1994).

**Enzyme linked immunosorbent assay (ELISA).** The ELISA test is reported to have a sensitivity of above 98%. The antigen is prepared from promastigotes of *L. donovani* and the test can be performed on serum, plasma or blood spots collected on filter paper. It is useful in the field owing to its simplicity, but false positives are not uncommon. The IFAT and DAT are preferable for laboratory diagnosis (Lainson & Shaw 1987).

A rapid immunochromatographic test using rK39 antigen produced from the membrane of amastigotes has given useful results in the diagnosis of VL (Tebourski *et al.* 1994; Singh *et al.* 1995).

**Direct agglutination test.** The DAT is a highly specific and sensitive test. It is cheap and simple to perform making it ideal for both field and laboratory use. The antigen is prepared from promastigotes of *L. donovani* and test can be carried out on plasma, serum, blood spots and whole blood. Antibody titres of 1 : 3200 are considered positive (El-Harith *et al.* 1995; El-Harith *et al.* 1996).

**Formol gel test.** The formol gel test has the advantage of being cheap and simple to perform. Serum obtained from about 5 ml of blood is mixed with one drop of 30% formaldehyde. A positive reaction is shown if the mixture solidifies and forms a white opaque precipitate within 20 min. A positive test cannot be detected until 3 months after infection and becomes negative 6 months after cure.

The test is nonspecific since it is based on detecting raised levels of IgG and IgM which also result from other infections such as African trypanosomiasis, malaria and schistosomiasis (Napier 1921) so it has very limited usefulness. Specific tests are preferred

**Molecular probes.** Recently, molecular probes, using kinetoplast DNA (kDaNA), ribosomal RNA (rRNA), mini exon derived RNA (medRNA) and genomic repeats have been evaluated. A probe has been developed to recognize the genomic DNA repeat Lmet2 that is specific for *L. donovani*. This probe has been used to diagnose infection in human patients with VL and in *Phlebotomus martini*, the sandfly vector of *L. donovani* (Wilson 1995). The early DNA probes were labelled with radioactive 32P, were easy to use and gave high signals with low background interference. However, these were not suitable as diagnostic kits, since 32P has a short half-life of about 14 days and, being radioactive, they presented safety problems. Recently, chemiluminescent hapten digoxigenin-labelled kDaNA probes have been developed. These probes are as sensitive as the 32P labelled probes (can detect as low as 100 parasites) and are stable enough to use in diagnostic kits, which are more practical in the field. However, high background signals make result interpretation difficult (Al-Masum *et al.* 1995; Simonsen & Dunyo 1999). The latest developments in molecular diagnostic technology have come with the advent of the polymerase chain reaction (PCR). PCR is able to amplify small amounts of DNA or RNA to larger usable quantities (Nuzum *et al.* 1995; Williams 1995; Wilson 1995).

Although PCR is able to detect a single copy of target DNA, repeat sequences are used to improve sensitivity. The early PCR assays required gel electrophoresis to interpret results, which was time consuming and not suitable for field use. An improved PCR-solution hybridization enzyme linked assay (PCR-SHELA) was developed and has been used to diagnose infection of *L. donovani* in patients in India, Kenya and Brazil with 90% sensitivity and 100% specificity (Nuzum *et al.* 1995). It has also been used as an epidemiological survey tool in Central America to detect the presence of *L. chagasi*. The PCR-SHELA
makes use of a biotin labelled probe; the product can be
detected using a spectrophotometer and the assay can be
performed in microtitre plates (Wilson 1995).

Helminths

Filariasis

Loa loa. Loiasis occurs principally in West Africa and
the Sudan.

Life cycle. The insect vector is the deer fly Chrysops spp.
which tend to inhabit swampy areas of the forest. After
being ingested when the fly takes a blood meal, the larvae
migrate to the fat bodies where they develop, before
migrating to the mouthparts when they reach the infec-
tive L3 stage.

The adults of this filarial parasite live in the subcuta-
neous tissues of their definitive hosts, which are humans
and other primates. The females measure from 20 to 70
mm in length and 425 μm in diameter, with the males
substantially smaller (20–34 mm long and 350 μm in dia-
meter).

Loiasis is often asymptomatic but episodic oedematous
subcutaneous swellings (Calabar swellings) and subcon-
junctival migration of an adult worm can occur. Larval
stages (microfilariae) are discharged into the circulation.

Laboratory diagnosis. Laboratory diagnosis is made by
demonstrating characteristic microfilariae in the blood.
The microfilariae, which are sheathed, are found in the
peripheral blood during the day with a peak between
1200 and 1400 h and in the lungs at night. Several meth-
ods are used to demonstrate and identify them, the most
useful involving concentration by filtering anticoagulated
blood through a nuclepore™ membrane of 5 μm-pore size
to capture all the microfilariae. Confirmation can be
obtained by staining the microfilariae with a combination
of Giemsa stain and Delafield’s haematoxylin to show the
presence of a sheath and nuclear extension to the end of
the blunt tail.

A significant increase in eosinophil numbers is seen
during the development of adult worms but is nonspecific
(Dennis 1993).

Sero logical techniques (ELISA or IFAT) utilizing extracts from whole worms or microfilariae as antigens,
can be used to demonstrate filarial antibodies. Antibody
detection is useful when the microfilariae cannot be
demonstrated but the assays are not species-specific and
exhibit cross-reaction with several other filarial species.

The development and use of specific monoclonal anti-
body s in an ELISA for the detection of circulating anti-
gens will probably increase specificity of the assays.Work
in Gabon (Toure et al. 1999) using serology for IgG4 and
nested PCR demonstrated that Loa loa specific DNA detec-
tion using nested-PCR amplification is the most sensitive
method in detecting loiasis, particularly with occult
infections without circulating microfilariae. Specific DNA
probes for filariasis are not as useful for patient diagnosis
as they are for epidemiological purposes.

Wuchereria bancrofti/Brugia malayi. The nematode
parasites Wuchereria bancrofti, Brugia malayi and Brugia
timori are the causative agents of human lymphatic filiar-
iasis.

Infection with these parasites most often consists of
asymptomatic microfilaraemia. Some patients develop
lymphatic dysfunction causing lymphoedema and ele-
phantiasis (frequently in the lower extremities) and, with
Wuchereria bancrofti, hydrocoele and scrotal elephantia-
sis. Episodes of febrile lymphangitis and lymphadenitis
may occur. Individuals newly arrived in endemic areas
can develop afebrile episodes of lymphangitis and lymph-
adenitis. An additional manifestation of filarial infec-
tion, mostly in Asia, is the pulmonary tropical
eosinophilia syndrome, with nocturnal cough and
wheezing, fever and eosinophilia.

Laboratory diagnosis. Based on finding microfilariae in
peripheral blood samples. Collection of the specimen is
dictated by the periodicity of the microfilaraemia, which
is determined by the geographical origin of the infections.
The microfilariae are seen nocturnally (2200–0200 h) in
most areas, except for a few parts of the South Pacific,
where Brugia timori occurs diurnally. Concentration
techniques and filtration can be used as described for Loa
loa. A study in south-west Ethiopia (Dennis 1993) claimed
that filtering 5 ml of day blood obviated the need to exam-
ine night blood, even though a high proportion of infected
persons had very low numbers of circulating microfilar-
iae.

A microhaematocrit tube technique using the QBC™
system has been used successfully to diagnose filariasis,
the microfilariae being seen above the buffy coat, with the
nuclei staining with AO under ultra violet light (Long et
al. 1990).

Three new commercially available tools for diagnosis of
Wuchereria bancrofti infections based on detection of
specific circulating antigens have been evaluated. These
tests are (1) The ICT card test for serum or blood speci-
mens (2) The TropBio ELISA for serum specimens. (3) The TropBio ELISA for filter paper specimens. The sensitivity for detecting microfilaraemic cases was 100% for all three. Furthermore, a significant correlation with microfilarial intensity was seen with the TropBio filter paper test (Simonsen & Dunyo 1999).

PCR-ELISA using digoxigenin labelled PCR products hybridized to a biotin-labelled probe followed by incubation in streptavidin coated microtitre wells and detection using antidigoxigenin peroxidase and ABTS is a specific and sensitive method improving on microscopic methods (Rahmah et al. 1998).

Other microfilariae found in peripheral blood are Mans onella ozzardi and Mans onella perstans, each of which can be detected by filtration of day blood samples using a 3-μm rather than a 5-μm pore membrane. They are non-sheathed microfilariae and are recognized by their smaller size and blunt tail with a prominent nucleus at the end (M. perstans) or sharply pointed tail free of nuclei (M. ozzardi).

Conclusion

Immunodiagnostic and molecular methods will become increasingly important for the detection and identification of blood parasites. However, light microscopy will still be required for the foreseeable future, and haematologists should maintain their proficiency in the morphological diagnosis of the common blood parasites of humans, especially malaria.

References


Sight **44**, 391–394.


