Manual on Visceral Leishmaniasis Control

World Health Organization
Division of Control of Tropical Diseases
Geneva, 1996

Overseas Development Administration
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INTRODUCTION

In March 1995 an international workshop took place at the London School of Hygiene and Tropical Medicine on the control of visceral leishmaniasis (VL). The workshop was initiated by the Overseas Development Administration (UK) as part of an ODA-funded research project, and was co-organised and co-sponsored by the World Health Organization and the Pan American Health Organization, with supplementary support from the European Commission and the International Development Research Centre (Canada).

The aim of the workshop was to:

(1) Produce a simple manual to the control of VL for the community, the physician, the diagnostician, the veterinary surgeon, the public health worker and the research scientist.

(2) Make the manual widely available in endemic regions and facilitate its translation into appropriate languages.

(3) Link efforts on control in the New and Old Worlds and to make the guide applicable in both.

There are many publications which concern the control of infectious diseases and this is certainly not the first that is relevant to VL. Two features of the international workshop and this report are, however, unusual:

(1) During the workshop interactive subgroups set down and answered the specific questions that confront those working at diverse levels on the control of VL.

(2) The text is divided into sections corresponding to the different roles of individuals.

This manual makes no attempt to present a scholarly debate on research enigmas. It is designed to provide a usable summary of the common practicalities of dealing with VL. To use the manual simply turn to the section of interest. Sections (1-9) are largely usable independently (except for the brief summaries of equipment at the end of each section) but where necessary follow the cross references to other sections.

Inevitably there will be errors and omissions in a first attempt at a manual of this type.
Please send suggestions for improvements or for other workshop topic to one of the following:

**Jorge Arias**
PAHO/WHO  
S.E.N. Lote 19  
70800-400 Brasilia, D.F.  
BRAZIL  
Tel: 55-61-312-6518; Fax: 55-61-321-1922  
Internet: jorge@opas.org.br

**Philippe Desjeux**
Division of Control of Tropical Diseases  
World Health Organization  
CH-1211 Geneva 27  
SWITZERLAND  
Tel: 41-22-791-3870/3903; Fax: 41-22-791-4777  
Internet: desjeuxp@who.ch

**Michael A. Miles**
Applied Molecular Biology Unit  
Department of Medical Parasitology  
London School of Hygiene & Tropical Medicine  
Keppel Street  
London, WC1E 7HT, U.K.  
Tel: 44-171-927-2340; Fax: 44-171-636-8739
1: VL AND PRIMARY HEALTH CARE

1.1 What are the diverse possible signs of human VL and PKDL?
(Paramedical or medical vigilance and response to suspected cases in the community)

The principal signs of VL are an enlarged spleen (Slide 1) and a prolonged irregular fever (Slide 2). Other signs and symptoms are loss of weight, pallor, an enlarged liver, enlarged lymph nodes, anaemia, cough and diarrhoea. These signs and symptoms may mimic those of malaria, typhoid, tuberculosis, schistosomiasis, malnutrition, tropical splenomegaly, histoplasmosis, and other diseases (see sections 2.1 and 2.2). Although VL occurs throughout its distribution in both children and adults, in the Americas and in the Mediterranean region (except in southern Europe) the disease is referred to as infant VL as the majority of cases occur in young children.

In endemic malarious areas kala-azar should be suspected when fever lasts for two weeks or more and no response has been achieved with anti-malarial drugs (assuming drug-resistant malaria has also been considered).

Post kala-azar dermal leishmaniasis (PKDL) is characterized by a maculopapular (patchy and raised) rash (Slide 3) and changes in skin colour (Slide 4). Late manifestations are plaques, papules or nodules (Slides 5 and 6). PKDL almost always occurs in patients previously treated for VL. It may be confused with lepromatous leprosy, fungal infections, diffuse cutaneous leishmaniasis (DCL) or other skin disorders.

1.2 What action should be taken with suspected patients?

Record the clinical history of the patient and the reasons for suspecting VL. The next step depends upon the structure of the health service of your country. If you have no means to confirm your clinical suspicion, you should refer the patient, without delay, to the next level for confirmation of the diagnosis. If it is possible, you should take samples from the patient and send them to a diagnostics laboratory (see sections 2.6 and 2.7).

1.3 What are the indicators that sandfly vectors are present?

Ask the local people if they are bitten by small flies (Slide 7) shortly after sunset or later during the night. Ask if there is a local name for such a fly. In some localities they are known as a serious biting nuisance (Slide 8), particularly at limited times of the year. At times sandflies can be found resting inside houses in the early evening hours. Attempts should be made to catch and keep suspect insects for later identification (Appendix 12).
1.4 **What immediate measures can be taken to reduce sandfly bites?**

Efforts should be made to reduce the number of bites by wearing appropriate clothes and, if available, by sleeping under fine mesh mosquito nets or preferably nets impregnated with a synthetic pyrethroid insecticide (Slides 9 and 10 and Appendix 3) (the impregnation of bednets by insecticides allows the use of bigger meshes). If it is known that sandflies are biting people inside the houses, the application of insecticide to the inner walls of the house (Appendix 12) should reduce exposure to sandfly bites. Since many species of sandflies which live in and around houses breed in organic rotting material, a community effort to keep the environment clean, particularly animal shelters (Slide 11), may be a useful intervention. When feasible, animal dwellings such as chicken sheds or pig sties, frequently infested with large numbers of sandflies (Slide 12) should be sprayed with insecticide to reduce breeding sites (Slide 13).

1.5 **What are the possible signs of canine VL?**

The initial stages of canine VL may be without obvious signs of disease (Slide 14). The earliest sign of VL in dogs is loss of hair, particularly around the eyes (Slide 15). As the disease progresses this becomes more pronounced. Dander, scaly lesions, and ulcers are common features (Slide 16). The dog is notably thin and becomes inactive. The lymph nodes are enlarged (the popliteal nodes at the back of the hind legs are the easiest to examine). The mucous membrane of the mouth and lips are pale and there may be shallow ulcers there or around the nose (Slide 17). In late stages, the claws are long and deformed (Slide 18) and there is a purulent discharge from the eyes (Slide 19). Keratoconjunctivitis may be apparent (Slide 20).

1.6 **What action should be taken with suspected dogs and what should be done about reporting their presence?**

If the dog is seriously ill and is suspected of having VL, the owner should be advised to have it destroyed humanely and without delay.

Records should be kept of the number of suspected dogs and where they can be found. The presence of these dogs should be reported through the regular health channels appropriate to your locality. Action against canine VL may be integrated with control of rabies and hydatid (see section 7.7). Domestic dogs that have become feral (free-running and wild) should be destroyed humanely and hygienically.

* In some endemic areas of human VL dogs are not known to be infected (e.g. in India, see Appendix 2)
1.7 What minimum or special equipment and services are required?

1.7.1 Notification of all *Leishmania* cases should be made obligatory by legislation.

1.7.2 Records of suspected or proven cases of human or canine VL should be made on standard forms of notification.

1.7.3 If no map of the local area is available one should be prepared showing houses and routes of access to them. All cases should be mapped to assist epidemiological studies and planning of control interventions.
2: VL AND THE PHYSICIAN
(Clinical diagnosis and referral)

2.1 What features are used to make a differential clinical diagnosis?

As an example these are the clinical features recorded for patients with VL in the Sudan, Brazil and India.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Sudan</th>
<th>Brazil</th>
<th>India</th>
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<tbody>
<tr>
<td>Fever</td>
<td>95%</td>
<td>95%</td>
<td>99%</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>95%</td>
<td>99%</td>
<td>98%</td>
</tr>
<tr>
<td>Uncomfortable spleen</td>
<td>85%</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Weight loss (wasting)</td>
<td>80%</td>
<td>98%</td>
<td>87%</td>
</tr>
<tr>
<td>Anaemia</td>
<td>75%</td>
<td>98%</td>
<td>96%</td>
</tr>
<tr>
<td>Lymph node enlargement</td>
<td>75%</td>
<td>30%</td>
<td>90%</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>70%</td>
<td>20%</td>
<td>30%</td>
</tr>
<tr>
<td>Cough</td>
<td>75%</td>
<td>40%</td>
<td>50%</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>60%</td>
<td>90%</td>
<td>98%</td>
</tr>
<tr>
<td>Epistaxis (nosebleed)</td>
<td>50%</td>
<td>30%</td>
<td>10%</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>40%</td>
<td>60%</td>
<td>50%</td>
</tr>
<tr>
<td>Vomiting</td>
<td>15%</td>
<td>infrequent</td>
<td>infrequent</td>
</tr>
<tr>
<td>Jaundice</td>
<td>5%</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Oedema</td>
<td>5%</td>
<td>40%</td>
<td></td>
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These clinical features are common to all endemic areas but some such as lymph node enlargement are far less frequent outside the Sudan and India.
The incubation period for VL is typically 2-6 months but may be shorter or much longer. The onset may be gradual or acute.

In many endemic areas malaria is the most common infection that can have a similar clinical presentation. VL should be considered in patients with a prolonged irregular fever, accompanied by other suggestive symptoms (above) when there is no response to anti-malarials and no malaria parasites can be found by repeated examination of blood films.

In all countries differential diagnosis must also consider typhoid (enteric fever), tuberculosis, AIDS, brucellosis, chronic hepatitis, cirrhosis, lymphomas and leukaemia. Massive splenomegaly may be a feature of VL, portal hypertension (due to cirrhosis and schistosomiasis) and malaria (due to hyperactive malarial splenomegaly).

Laboratory diagnosis, if available, to detect leukopenia (85% of patients in the Sudan), thrombocytopenia (75% of patients in the Sudan) and positive serum antibody (95% of patients who do not have AIDS) is valuable for confirming clinical diagnosis (see section 3).

2.2 **What atypical clinical presentations might occur?**

Occasionally the clinical presentation is atypical, without splenomegaly but with fever, wasting, diarrhoea, cough, or combinations of these. In West Bengal, India, generalized lymph node enlargement without visceral involvement was reported.

Simple cutaneous lesions (leishmanioma) may occasionally precede, accompany or follow VL. PKDL may occur before VL in rare cases, or in Africa begin during treatment.

Infections due to *Leishmania infantum*/*Leishmania chagasi* (Appendix 2) are apparently often asymptomatic. Most individuals who have evidence of exposure to *Leishmania*, with a positive leishmanin skin test or positive serology (typically 3 - 30% of the population in endemic areas) do not recall having a clinical illness. Infection with *Leishmania donovani* (Appendix 2) is thought to be more frequently clinically apparent than infection with *L. infantum/L. chagasi*, but subclinical VL is always more common than clinical VL.

2.3 **When is clinical diagnosis a basis for initiating drug treatment?**

When laboratory facilities are not available, as clinical (symptomatic) VL (see 2.1 and 2.2 above) is not benign or self-limiting and it is usually fatal if untreated, appropriate treatment should be given.
2.4 **What complications and secondary infections might occur in VL?**

Secondary infections are common, and include pneumonia, bronchial infections, tuberculosis, malaria, diarrhoea or dysentery, viral infections, bacterial skin infections, otitis media (inflammation of the middle ear), and cancrum oris (mouth lesions).

Thrombocytopenia may cause epistaxis (nosebleed), or bleeding from other sites, and this may precede death.

*Leishmania* enteritis may be a cause of diarrhoea and malabsorption and pulmonary involvement may mimic pneumonia.

Death is mainly due to secondary infection, or haemorrhage.

2.5 **What immediate clinical responses are appropriate?**

(a) A patient should ideally be referred to a centre where diagnostic facilities, drugs and expertise in the treatment of VL are available. If lack of resources or other logistic problem prevent this, treatment should be given even in remote areas as soon as possible (see section 4).

(b) Coexisting malaria, anaemia, bacterial infections and tuberculosis should all be treated simultaneously.

(c) The patient should be provided with adequate food during treatment.

(d) The physician should make an enquiry about other cases of VL in the family and village, and notify the relevant health authorities about the existence of VL.

2.6 **How can samples be collected for serological diagnosis?**

(a) **For the direct agglutination test (DAT):**

Puncture the patient's finger with a lancet (after cleaning the skin thoroughly with a 70% alcohol swab), turn the finger downwards and collect two drops of blood (each making a separate blood spot of 1 cm diameter) on to Whatman No. 3 filter paper. Make sure the finger never touches the filter paper during the operation.

Label the filter paper with the patient's name, code number and date (Slide 21).

Allow the blood spots to dry for few hours at room temperature and then store them dry singly in a plastic envelope or sealed container, or with clean dry paper
separating filter paper sheets.

The filter papers may be stored at room temperature for one week, for months in a 4°C fridge, or for years in a -20°C freezer. One blood spot should be used for diagnosis and one saved for future reference or repeat testing.

The blood spot collection method is also sometimes used for other serological tests, such as the indirect immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (see (b) below).

Serum ((b) below) can also be used for the DAT and may give more accurate results.

(b) *For the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA):*

Take about 1 to 2 ml of blood from the vein with a sterile syringe and needle into a sterile container without anticoagulant. Allow the blood to clot, take off the serum, discard the red cells, and store the serum at 4°C in a fridge, or at -20°C if longer storage is needed.

(c) *For the formol-gel slide test:*

Serum can also be used for this test. It is not specific for VL but detects hyperglobulinaemia, which is often associated with VL.

2.7 *How can samples be collected for parasitological diagnosis?*

(a) *Bone marrow aspirate:*

Under local anaesthetic, aspirate a sample (up to 1 ml) of bone marrow from the iliac crest or the sternum using a sterile bone marrow aspirate needle and a 10 ml syringe. Immediately make thin smears of the aspirate on at least three glass microscope slides. Allow smears to air dry (or dry rapidly by rubbing the back of the slide with a finger to slightly warm the glass and drive off moisture), fix with 100% methanol (methyl alcohol), dry, label and store protected from insects until staining. Stain with Giemsa or May-Grünwald Giemsa (section 3). Examine at least 1000 fields per slide under oil immersion (100X magnification) preferably around the edges of the preparation.

If culture medium is available carefully inoculate culture tubes with one or two drops of the aspirate, using aseptic precautions (swab the culture tube rubber cap with 70% alcohol and inoculate through the cap) to avoid contaminating the
medium. The medium should be NNN (Slide 22), blood agar, or sloppy Evan's medium (Appendix 4). Keep culture tubes at room temperature (25°C optimal). In very hot countries steps must be taken to prevent the cultures from overheating, e.g. cover with a damp towel or place near a ventilator to keep the temperature down.

(b) Lymph node aspirate (Slide 23):

Inguinal and epitrochlear lymph nodes are most convenient. Grasp the lymph node between thumb and fingers, introduce a 21 gauge needle attached to a 5 ml syringe into the lymph node, milk (gently press the lymph node several times) or move the needle backwards and forwards several times, and remove it. Make smears (and cultures if available, and if there is sufficient material) as in (a) above.

(c) Splenic aspirate (Slide 24):

**THIS PROCEDURE MUST NOT BE PERFORMED WITHOUT TRAINING AND EXPERIENCE: IT MAY LEAD TO FATAL HAEMORRHAGE IF DONE INCORRECTLY.**

First ensure the patient is not at risk of bleeding, and that facilities are on hand to deal with bleeding. The platelet count should be greater than 40,000 and prothrombin time greater than 50%. (See WHO technical report series 793 annex 4 for a full description of requirements.) In brief, fix the spleen between thumb and forefinger of the open hand. Introduce a 21 gauge needle attached to a 5 ml syringe just through the skin, and draw back the plunger to the 1ml mark to apply suction. Maintaining suction throughout and with a very rapid in and out movement, push the needle into the spleen to the full needle depth (3 cm) and then withdraw it immediately and completely. The entry and exit trajectory of the needle must remain the same and should be perpendicular to the spleen.

*Notes:*

Material from splenic and lymph node aspirates will be very scanty, and the whole specimen may be needed to prepare the smear. The contents of the syringe may have to be briskly squirted out of the syringe onto the slide in order to obtain an adequate specimen. The syringe and needle may then be rinsed in culture medium for inoculation of culture tubes.

If it is necessary to send diagnostic samples (e.g. live cultures) to a reference centre through the mail, local legal requirements must be checked and followed.

2.8 **What minimum or special equipment and services are required for clinical**
diagnosis and collection of samples for laboratory diagnoses?

An examination area; clinical thermometer; microscope; microscope slides; Giemsa stain; bone marrow aspiration needles; disposable syringes (5 ml and 10 ml); disposable needles; local anaesthetic; blood lancets; filter paper; plastic bags or boxes; plastic screw-top blood and serum containers; pasteur pipettes; access to serology locally or by referral of samples (access to treatment for all cases detected - see section 4).

2.9 To whom should cases of VL be reported?

A diagnosis of VL should be followed by notification to the local, regional and national health authorities, irrespective of whether the case of VL is parasitologically confirmed or not. Details should include the patient’s name, age, sex, locality (address), the results of tests if known, whether the patient has travelled to an endemic area (where, when and for how long), whether the patient is immunosuppressed, and whether the patient is part of a cluster or outbreak.

2.10 What other action should the physician take?

The physician should try to determine whether other cases of VL exist in the same family or/and in neighbouring households, by active case finding, and should communicate with authorities in neighbouring areas, to discuss the extent of the problem. In the case of VL with a (canine) reservoir (zoonotic VL), the physician should assist veterinary and environmental health teams.

In all instances the physician should try to ensure that patients diagnosed as having VL receive a full course of treatment at the right dose and without any interruption to prevent drug-resistance, relapse, and PKDL (see section 4).

Case histories of VL (immunocompetent)

Case History - VL - 1

A girl, aged 13 years, from a VL endemic area in Kenya was admitted to a district hospital complaining of fever for three months, left sided abdominal pain, tiredness and loss of weight.

Examination revealed pallor and severe emaciation, 12 cm palpable splenomegaly, 5 cm palpable liver and generalized lymphadenopathy.

Investigations showed: Hb 6.6 gm/dl WBC 2x10^9/l, and a negative blood film for malaria. The DAT titer was 64,000. A lymph node aspirate was positive for Leishmania.

The patient received pentavalent antimony, 20 mg/kg body weight daily for 30 days, multivitamins and iron. The fever subsided and the spleen size regressed by 2 cm in the first
week of treatment. The patient started to gain weight and her general condition improved steadily. She was discharged after completion of treatment in good condition. The spleen remained just palpable for 12 months, but there were no symptoms of relapse.

Case history - VL - 2

A 10 year old boy from Sudan was admitted to hospital complaining of fever, diarrhoea and epistaxis for two weeks.

On examination: the patient was pale and wasted. The spleen was just palpable but the liver and lymph nodes were not enlarged. Investigations showed: pancytopenia, a negative blood film for malaria. The DAT titer: 102,400. Bone marrow smear was positive for *Leishmania*. The patient was treated with pentavalent antimony (20 mg/kg body weight) daily for 30 days, multivitamins and iron. After one week of treatment the fever subsided and the patient was feeling better with increasing appetite. The patient was discharged in good condition after 30 days of treatment. He returned for follow-up at 3 and 6 months and continued to be in good health.
3: VL AND LABORATORY DIAGNOSIS

3.1 How is microscopy performed for parasitological diagnosis?

Smears of aspirates or biopsies (see section 2) arriving from public health centres or family doctors should all ideally be examined on the day of arrival. If slides have to be kept overnight they should be stored dry in closed containers at room temperature.

If smears are prepared from patients at the laboratory they should be dried rapidly (e.g. by rubbing the back of the slide with a finger to slightly warm the glass and drive off moisture) and then fixed immediately with absolute (100%) methanol (methyl alcohol) for one minute. The methanol must be stored in tightly closed bottles to prevent absorption of water.

Stain the films with Giemsa as described in Appendix 5.

Examine the Giemsa-stained smears using a 100X oil immersion objective. Leishmania amastigotes are very small round or oval organisms about 3 μm x 5 μm found inside or outside phagocytic cells (macrophages). Each amastigote contains a red-mauve nucleus, a smaller more deeply staining red-mauve kinetoplast, and pale blue cytoplasm. The particular diagnostic feature to look for in VL is the presence of nucleus and kinetoplast in these organisms (Slides 25, 26 and 27).

In about 50% of patients that are severely immunosuppressed, such as HIV co-infected individuals, Leishmania amastigotes may be found in thin films or thick smears of peripheral blood.

The sensitivity of parasitological examination can often be increased by aseptically placing aspirate or biopsy material into culture medium and then examining the medium after several days. [The preparation and inoculation of culture media is described in Appendix 4.] This has the disadvantage that an immediate diagnosis cannot be provided. Also, if laboratory staff are not skilled in the preparation of the medium and inoculation of the medium with aspirate and biopsy material, cultures are very prone to contamination with bacteria or fungi. Leishmania usually grow in culture as free-swimming, flagellated promastigotes, although they may also divide as clumps of amastigotes (Appendix 4, Slide 28).

3.2 What haematological signs may be associated with VL?

Haematological tests in the laboratory that are useful for detecting signs of VL are the haematocrit to determine packed cell volume (PCV), determination of haemoglobin (Hb),
white cell count (Appendix 5) and determination of total serum protein.

These tests may indicate:

Anaemia, especially in severe cases of VL.

Reduced white cell counts (leukopenia) with total white cell counts down to 2.0 \times 10^9/l.

An increase in serum total protein (Slide 29).

There may also be a very high erythrocyte sedimentation rate (ESR) and reduced numbers of platelets (thrombocytopenia) leading to a prolonged blood-clotting time.

3.3 **What serological tests are useful for diagnosis of VL?**

Available serological tests are the formol gel test (aldehyde test) in tubes or on slides (useful in the absence of any other), the direct agglutination test (DAT), the indirect immunofluorescence test (IFAT) and the enzyme-linked immunosorbent assay (ELISA or Dot-ELISA). These tests are described fully in Appendices 6 (formol gel test), 7 (DAT test), 8 (IFAT) and 9 (ELISA) (Slides 30, 31, 32 and 33).

The formol gel test and DAT (given a reliable standardised source of DAT antigen) can be performed outside laboratories in primary health care centres. The IFAT and ELISA tests require laboratory facilities and more sophisticated equipment, although rapid ELISA or dipstick tests are being developed for use in primary health care centres or in the home.

3.4 **What diagnostic methods should be used to follow-up patients who have been treated?**

In treated patients who show no clinical and/or haematological improvement or who have a clinical relapse, parasitological diagnostic tests should be repeated to determine if *Leishmania* are still present. Parasitologically positive patients will require re-treatment, possibly with a modified course of treatment or a second line drug or combination of drugs (see section 4).

For patients who have shown good clinical improvement a positive leishmanin skin test (Montenegro; Appendix 10, slide 34) provides confirmation of cure.
3.5 **What minimum or special equipment and services are required for laboratory diagnosis?**

For microscopical examination of stained smears: microscope with (50X) or 100X oil immersion objective, microscope slides, staining trays, syringes (10 or 20 ml), needles, sodium chloride, disodium hydrogen phosphate, Giemsa stain.

Other equipment and services for the parasitological, serological and haematological tests are described in the Appendices (4 - 10).
4: TREATMENT OF VL AND SIDE EFFECTS

4.1 What are the criteria, dependent on available resources, for the initiation of treatment?

Other causes of the clinical picture, such as malaria, should be excluded (see section 2).

Ideally, all VL cases should be confirmed parasitologically. In field conditions, however, clinical features and a positive serological test for *Leishmania* (DAT test; see section 3) or, to a lesser degree, a positive formol gel test (see section 3) and, if available a negative leishmanin test justifies starting treatment (Slide 35)(the leishmanin skin test is negative during active VL).

In remote areas where neither parasitological or serological diagnosis is possible a trial of treatment may be necessary, if malaria or other causes have been excluded. A response to antimonials should be seen within a week to ten days, with a fall in temperature, increased feeling of well-being and an increased appetite.

When clinical (symptomatic) VL occurs (see 2.1 and 2.2 above) it is not benign or self-limiting and must be treated, as it is usually fatal if appropriate treatment is not given.

4.2 How should treatment with antimonials be given? What are the side effects and contra-indications? How much does the treatment cost?

*Treatment*

The World Health Organization recommends treatment with pentavalent antimony at 20 mg pentavalent antimony/kg/day for 30 days. Previously, an upper limit of 850 mg pentavalent antimony/day was advised, but more recently this has been revised, and there is no upper limit on the daily dose of 20 mg pentavalent antimony/kg. Treatment may be given as a once daily intramuscular injection (which is preferable for children) and in adults a larger volume of pentavalent antimony may require intravenous injection or infusion. Intravenous injection should be given over 5 - 10 minutes and infusion should be diluted in 50 - 100 ml of 5% dextrose solution. In severely wasted children, the intramuscular injection may have to be divided among multiple sites. The following three antimonial preparations are currently available:

(1) Pentostam (sodium stibogluconate) is available from Wellcome, U.K. It contains 100 mg pentavalent antimony/ml.
(2) Glucantime (meglumine antimoniate) is available from Rhône-Poulenc, Rorer/Specia, France and Rhodia Farma, Brazil: it contains 85 mg pentavalent antimony/ml.

(3) Sodium antimony gluconate (identical to sodium stibogluconate) is available in India, from Albert David Limited, Delhi, from Stibanate Company Limited, Calcutta and from Anoco Pharmaceuticals, Patna: it contains 100 mg pentavalent antimony/ml.

Side effects to antimony treatment

In clinical practice minor side effects are common, moderate side effects are uncommon and severe side effects very rare. The commonest side effects are pain at the injection site, muscle pain (myalgia), joint pain (arthralgia), loss of appetite and nausea. These symptoms are relatively mild and myalgia and arthralgia may be controlled by paracetamol. QT segment changes may occur on the ECG, and therefore in ideal circumstances ECG monitoring before treatment and weekly during treatment should be performed, but clinically important arrhythmias or heart failure are very unusual. In ideal circumstances weekly monitoring of hepatic and renal function and amylase should be undertaken, though these rarely give rise to symptomatic illness.

There are no absolute contra-indications to pentavalent antimony treatment, and even severely ill patients should respond. Pentavalent antimony is not contra-indicated in pregnancy. If underlying cardiac, renal or hepatic disease is present, the patient should, ideally, be carefully monitored during treatment and other drugs should be considered.

Cost

The cost of treatment is very high: for a 60 kg patient the cost of Pentostam will be approximately $150; Glucantime would cost about $120 and sodium antimony gluconate approximately $16.

Other aspects of treatment

There may be considerable non-drug costs in the treatment of VL: hospital admission, additional food, transport, and loss of income from the carers. In ideal circumstances, all patients should be treated in hospital.

In practice, the economic burden of hospital treatment means that, in countries that have less funds allocated for health care, uncomplicated VL cases may be treated by daily injections at a dispensary or in the home by visiting health workers, and selected cases referred for hospitalization. Criteria for referral to hospital include: severe anaemia (haemoglobin less than 5 grams/100 ml), severe or prolonged diarrhoea, severe wasting,
or non-response to treatment.

Simultaneous infections with malaria, HIV or tuberculosis should be suspected if the clinical response is poor, and patients who have tuberculosis and VL must be referred to hospital for simultaneous treatment. Unresponsive or relapsed cases should always be referred to hospital.

4.3 How should unresponsive or relapsed cases be treated?

Patients who are unresponsive to a course of pentavalent antimony or who relapse after treatment should never be retreated without parasitological confirmation of the diagnosis, and response to treatment must be monitored parasitologically. This requires that the patient be referred to a specialist centre.

Patients who have been referred for a relapse or unresponsive VL should always be investigated for concomitant infection with tuberculosis (by sputum smear, and, if available, by chest x-ray), malaria (by microscopy of Giemsa stained blood films), amoebic dysentery or amoebic liver abscess, or HIV. Occasionally, empirical treatment for typhoid fever may also be required.

Notes on second courses of treatment:

(a) In many cases, the exact dose, duration and quality of pentavalent antimony used for the first course may not be known, particularly if the treatment was done in a remote area. In these circumstances a supervised course of pentavalent antimony, 20 mg/kg daily for 30 days should be given.

(b) If the patient is clinically unresponsive to a supervised course of pentavalent antimony after two weeks of treatment, or is parasitologically unresponsive at the end of treatment (with bone marrow or other aspirates still positive for parasites after 30 days of treatment) then a second line drug or drug combination should be used (see below).

(c) The practice of using intermittent courses of pentavalent antimony with drug-free periods in between is illogical and should be discouraged. It may lead to the emergence of pentavalent antimony-resistant disease.

Second-line drugs

(a) Amphotericin B (Fungizone, Squibb). A suitable regimen is 0.5 mg/kg by intravenous infusion daily, or on alternate days, until a total dose of 20 mg/kg
has been given. In some areas a lower total dose has been successful, for example in India a total dose of 7 mg/kg is reportedly successful. The major side effect of amphotericin B is renal impairment, and renal function should be monitored weekly during treatment. Renal impairment can be reduced by pre-hydrating the patient with an infusion of normal saline. If a rise in urea and creatinine occur, the interval between doses should be lengthened. Other side effects include fever and anaemia.

(b) Aminosidine (paromomycin; Gabromicina, Farmatalia). Aminosidine is an anti-leishmanial aminoglycoside which may be synergistic with pentavalent antimony. A suitable regime is pentavalent antimony 20 mg/kg daily for 30 days plus aminosidine at 15 mg/kg daily for 30 days. The two drugs are given by separate injections in two separate sites. Aminosidine may cause renal impairment and urea or creatinine should ideally be monitored weekly during treatment. It might also affect the auditory nerve and cause high-tone deafness.

(c) Other drugs which have been used in repeated relapses and unresponsive cases are: Pentamidine isethionate, 4 mg/kg on alternate days for 11 weeks; pentavalent antimony + allopurinol at 20 mg/kg/day in divided doses; liposomal amphotericin B (AmBisome), total dose 20 - 30 mg/kg given over 10 - 20 days; pentavalent antimony + gamma-interferon. Pentamidine may cause hypoglycaemia, diabetes, renal impairment and pancreatitis. AmBisome is effective with very little toxicity but is very expensive. Experience has shown that it can be given in 5 or more doses of 3 - 4 mg/kg over a 10 day period, with excellent results.

4.4 **What supportive measures are required during treatment?**

Patients should receive adequate nutrition. Vitamin supplements and iron may be added.

Treat dysentry with antibiotics and hydration.

Treat pneumonia with antibiotics.

Maintain oral hygiene to prevent mouth infections (cancrum oris) and rapidly treat cancrum oris, should it occur, with metronidazole and penicillin.

Maintain skin hygiene and treat skin sepsis.

Treat malaria and/or tuberculosis if present.

Very occasionally, blood transfusion may be required for severe anaemia or bleeding due to thrombopenia.
Vitamin K may be of benefit in severe epistaxis.

4.5 **How can cure be evaluated?**

At the end of treatment (day 30) clinical assessment should show weight gain, a regressing spleen, and the patient should have been without fever for the last two-three weeks. The haemoglobin and white blood cell count should be rising.

Parasitological confirmation of cure is not routinely necessary and may be reserved for cases where response is in doubt.

Parasitological confirmation of cure is essential, however, in the treatment of relapses.

After treatment the patient should be reviewed at 1, 3, 6 and 12 months. Patients should be told to report if they develop symptoms of VL or a skin rash (PKDL).

Good clinical progress would be: no recurrence of fever; weight continuing to rise; spleen size continuing to regress. In addition, the haemoglobin should be rising.

The leishmanin skin test should become positive in 80% of patients 12 months after successful treatment.

A persistently enlarged spleen is no cause for concern provided the patient's other symptoms are improving, and residual enlargement of the spleen may persist for months or years after successful treatment.

Lymphadenopathy, which is common in some places, e.g. Sudan, India, may persist for months or years after successful treatment.

*Relapse*

After a complete course of effective treatment in immunocompetent patients less than 5% of patients will relapse.

Clinical features of relapse are a fever, weight loss, and an enlarging spleen size.

Relapse of VL is most likely to occur within the first 3 months after treatment, and is most unlikely after 12 months.
4.6 **How is post-kala azar dermal leishmaniasis (PKDL) treated?**

PKDL is treated with pentavalent antimony, in the same dose and by the same routes as VL. A dose of 20 mg pentavalent antimony/kg/day for 4 months or longer is used in Indian PKDL. In African (Ethiopia, Kenya and Sudan) PKDL 2 to 3 months of treatment may suffice. Once lesions improve clinically treatment may be stopped, as PKDL very rarely relapses.

4.7 **What minimum or special equipment and services are required for treatment?**

Adequate supplies of pentavalent antimony, sterile syringes and sterile needles are required for treatment of uncomplicated VL at a dispensary or in the home by a visiting health worker.

Hospital services with access to parasitological and serological diagnosis (and if possible leishmanin skin test) are required for treatment of unresponsive or relapsed cases (see section 3 and relevant appendices). Additional hospital services are required to support patients with HIV co-infection or other co-infections (see 4.3 above).
5: VL AND IMMUNOCOMPROMISED PATIENTS

5.1 **What types of immunocompromised patients may be affected?**

Any condition reducing the cellular immune response will make VL more likely, more atypical and more resistant to treatment.

HIV infected patients are by far the most common group of immunocompromised patients that suffer VL co-infection, especially in Europe. European patients with HIV/VL co-infection have low CD4 (helper T lymphocytes) counts: 97% of patients have CD4 cell counts of less than 500/μl and 80% have CD4 counts of less than 200/μl.

Other immunocompromised patients that may be more susceptible to VL are those receiving corticosteroid and immunosuppressive therapy, patients with lymphoma, with leukaemia, with chronic hepatitis, renal transplant, sarcoidosis, Crohn's disease, systemic lupus erythematosus, thymectomised patients or those with ulcerative colitis.

In endemic areas for VL in Africa, South America and India severe malnutrition predisposes to VL and this may be a result of reduced ability to produce a cell mediated immune response.

5.2 **What atypical clinical presentations of VL might be seen in immunocompromised patients?**

The following table summarises clinical features of VL in 96 European patients with HIV co-infection:

<table>
<thead>
<tr>
<th>Feature</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>29-33</td>
</tr>
<tr>
<td>Male</td>
<td>86%</td>
</tr>
<tr>
<td>Fever</td>
<td>88%</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>79%</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>78%</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>50%</td>
</tr>
</tbody>
</table>
Bi- or pancytopenia 79%
Hypergammaglobulinaemia 72%
*Leishmania* serology positive 50%

CD4+ T cells/μl

- >500 3%
- 500-200 17%
- <200 80%

Atypical clinical features are seen in some patients with HIV/VL co-infection.

The infection may be present in the skin with cutaneous, diffuse cutaneous or mucocutaneous leishmaniasis. The infection may be widely disseminated in the viscera and other organs may be involved. Diarrhoea, cough and difficulty in swallowing (dysphagia) may be common. Cryptosporidiosis (*Cryptosporidium* infection) may also cause diarrhoea in patients with HIV infection.

Patients with HIV/VL co-infection may have nodular or ulcerative lesions on the tongue (slide 36), oesophagus, stomach, rectum, larynx and in the lungs. Parasites may be seen in numerous sites with or without the presence of lesions.

*Leishmania* may be found in bone marrow aspirates and unexpectedly in skin biopsies (including biopsies of other lesions such as those of Kaposi's sarcoma) and in normal skin; duodenal biopsies; rectal biopsies; broncho-alveolar lavage; and in peripheral blood neutrophils.

In immunocompromised patients, VL may be rapidly progressive, resembling bacterial sepsis. Alternatively, VL may be unusually slowly progressive with a few, non-specific symptoms, and in some patients, VL may be entirely asymptomatic.

In HIV patients a clinical picture similar to that of VL may be caused by other opportunistic infections; disseminated mycobacterial infection; cytomegalovirus (CMV); salmonellosis and disseminated fungal infections (cryptococcosis). Lymphoma may also be suspected.

30% of HIV/VL patients will die during treatment or within one month after treatment.
The mean survival with optimal treatment is only 12 months. Only 16% will survive for more than 3 years. Death is seldom due to VL alone. A sterile cure cannot be achieved by any drug, and relapse is almost inevitable. The time to relapse is usually 3 - 6 months, with successive relapses becoming less typical and less acute, but occurring more frequently.

5.3 What modifications are required to the methods of diagnosis for immunocompromised patients?

In immunocompromised patients parasitological diagnosis has increased sensitivity. Microscopy of bone marrow aspirates is reported to be 94% sensitive in immunocompromised patients and culture of bone marrow in an appropriate culture medium (Appendix 4) is reported to be 100% sensitive. Sometimes it can be difficult and has to be repeated.

Immunocompromised patients may have circulating amastigotes in neutrophils in the peripheral blood (blood parasitemia). Microscopy of blood films may be useful and is said to be positive in 50% of patients. Microscopy of buffy coat concentrated from blood by centrifugation (e.g. in an haematocrit centrifuge) increases sensitivity further and culture of buffy coat is said to be positive in 70% of immunocompromised patients.

In contrast, because of the compromised immune response, serology has low sensitivity in immunocompromised patients.

20 - 40% of HIV/VL patients may be serologically negative and diagnosis must therefore rely on the parasitological detection of Leishmania.

5.4 What modifications might be needed in treatment and supportive measures for immunocompromised patients?

Patients with HIV/VL should be treated as outpatients wherever possible.

HIV/VL coinfection patients treated as outpatients, however, must be considered as highly infectious reservoirs in areas of active transmission because of the presence of amastigotes circulating in the blood.

Alternative treatments are as follows:

(1) Pentavalent antimony at 20 mg/kg/day (without an upper limit on the dose) for 30 days (see section 4).

83% of the cases respond positively but 50% of them relapse from one to four
times between one month and 36 months after treatment. Toxicity of pentavalent antimony is greater in HIV patients and includes pancreatitis, blood abnormalities and drug allergies. Secondary effects often lead to interruption of the treatment.

(2) Amphotericin B at 0.5 mg/kg by intravenous infusion daily or on alternate days until a total dose of 20 - 30 mg/kg has been given. Side effects of treatment by amphotericin B may be impairment of renal function, fever, chills, anaemia, low potassium levels in the blood (hypokalemia), or low magnesium in the blood (hypomagnesemia). These side effects may be minimised by pre-treatment with paracetamol or antihistamines and pre-hydration with oral fluids or intravenous saline.

After treatment, parasitology need only be repeated where there is no improvement in the clinical picture due to VL.

(3) Maintenance treatment: the only treatment used to prevent relapse in HIV patients with VL. (maintenance treatment) is at present pentamidine isethionate intravenously at 4 mg/kg once a month or 2 mg/kg every two weeks (this will also provide adequate prophylaxis against Pneumocystis carinii). The efficacy of this maintenance treatment has not yet been clearly established.

In the current unsatisfactory chemotherapy situation, the most important objectives are to maintain a good quality of life for the patients, prevent relapses and avoid life-threatening infections.

5.5 What minimal or special equipment and services are required for the management of VL in immunocompromised patients?

The equipment and services are those described in sections 2, 3 and 4. In addition a reliable and rapid test for HIV infection must be available.

Case histories of VL (immunocompromised)

Case history - VL (immunocompromised) - 1

A 30 year old man addicted to heroin was admitted because of fever, cough and blood in the sputum (haemoptysis). He had a long history of chronic hepatitis, and AIDS had been diagnosed six months before, when cerebral toxoplasmosis occurred. He was disoriented and lethargic on admission. The skin was brown and dry, and there were a few petechiae on the lower limbs. He appeared to be severely malnourished, and his muscles were wasted. He had oral candidiasis and generalized lymphadenopathy. No visceral enlargement was felt.

He was pancytopenic, with a leukocyte count of 0.9 x 10⁹/l, Hb of 7g/dl and platelet
count of $11 \times 10^9/l$. The aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase (ALP) levels were slightly elevated. Laboratory tests also revealed the following values: serum protein, 55 g/l; CD4 lymphocytes, 8/µl; CD8 lymphocytes, 295/µl; and CD4/CD8 lymphocyte ratio, 0.03. An indirect immunofluorescence assay for antibodies to *Leishmania* was positive (titer, 1:80). A bone marrow aspirate contained many *Leishmania* amastigotes.

Treatment with meglumine antimoniate (20 mg/kg of pentavalent antimony) plus interferon gamma (four million units/day subcutaneously) was given over four weeks. After three days of therapy, he was afebrile and his general condition began to improve. At the end of therapy, pancytopenia had subsided, and a culture and smear of a newly obtained bone marrow aspirate were negative for *Leishmania* organisms. He died a few weeks later of cerebral lymphoma.
6: VL AND THE VETERINARY SURGEON

6.1 How can a differential clinical diagnosis of canine VL be made?

The following table summarises the differential diagnosis of canine VL:

<table>
<thead>
<tr>
<th>Signs of VL</th>
<th>Other possible causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>bleeding in the muzzle area</td>
<td>trauma</td>
</tr>
<tr>
<td>lymphadenopathy</td>
<td>lymphoma; leukaemia</td>
</tr>
<tr>
<td>ulcers/nodules on the skin or around the mouth</td>
<td>viruses; other infections or traumas</td>
</tr>
<tr>
<td>subcutaneous nodules</td>
<td>sebaceous cysts</td>
</tr>
<tr>
<td>dermatitis and loss of hair (depilation)</td>
<td>fleas; other ectoparasites (e.g. scabies); food induced inflammation of the skin (dermatitis); adrenal disease</td>
</tr>
<tr>
<td>elongated deformed nails (onychogriposis)</td>
<td>lack of use of claws (e.g. due to confinement in an apartment)</td>
</tr>
<tr>
<td>anaemia</td>
<td>poor nutrition; endoparasites; food poisoning; ticks; fleas</td>
</tr>
<tr>
<td>emaciation</td>
<td>loss of appetite (anorexia); starvation; endoparasites</td>
</tr>
</tbody>
</table>

6.2 How can samples be collected from dogs for serological diagnosis?

(a) For the direct agglutination test (DAT):

Puncture the ear margin with a lancet and collect two drops (each making a separate blood spot of 1 cm diameter) on to Whatman No. 3 filter paper.

Label the filter paper with the dog's name, code number and date.

Allow the blood spots to dry at room temperature and then store them dry singly in a plastic envelope or sealed container, or with clean dry paper separating filter paper sheets.
The filter papers may be stored at room temperature for one week, for months in a 4°C fridge, or for years in a -20°C freezer. One blood spot should be used for diagnosis and one saved for future reference or repeat testing.

The blood spot collect method is also sometimes used for other serological tests, such as the indirect immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (see (b) below).

Serum ((b) below) can also be used for the DAT and may give more accurate results.

(b) **For the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA):**

Take about 1 to 2 ml of blood from the vein with a sterile syringe and needle into a sterile container without anticoagulant. Allow the blood to clot, take off the serum, discard the red cells, and store the serum at 4°C in a fridge, or at -20°C if longer storage is needed.

(c) **For the formol-gel slide test:**

Serum can also be used for this test. It is not specific for VL but detects hyperglobulinaemia, which is often associated with VL.

6.3 **How can samples be collected from dogs for parasitological diagnosis?**

Samples can be collected from the following sites for parasitological examination:

(a) **A lymph node:** any superficial lymph node may be used but the lymph node behind the knee (popliteal lymph node) or the lymph node in front of the scapular (pre-scapular lymph node) are preferred. The lymph node is punctured with a sterile needle (Slide 37). Grasp the lymph node between thumb and fingers, introduce a 21 gauge needle attached to a 5 ml syringe into the lymph node, milk (gently press the lymph node several times) or move the needle backwards and forwards several times, and remove it. The lymph node aspirate should be smeared on to a clean, glass slide and air-dried rapidly, e.g. by rubbing the finger on the back of the slide to produce heat and drive off the moisture in the sample. It is essential that no blood is taken up during the gland puncture, or if it is, that it is not used in preparing the smear.

(b) **The bone marrow:** preferably by aspiration from the tip of the sternum with a 5 or 10 cc syringe and a 20-21 gauge needle. A smear of the aspirate is prepared on a clean glass slide and air-dried (as above for the lymph node aspirate).
(c) *The skin:* by biopsy using a local anaesthetic. A small sample (e.g. 2 mm x 2 mm and 2 mm deep) is taken with sterile scissors and forceps or with the aid of a sterile skin punch. The skin biopsy is blotted on clean, dry filter paper to remove all excess blood. An impression smear is made by pressing the internal face of the biopsy on to a clean glass slide, and the smear is then air-dried. Biopsies are best taken from any areas of skin that look altered or abnormal (e.g. with hair loss, discoloration, flaking or unusual roughness). The muzzle, the border of the ear and base of the tail are often affected and used for biopsy.

(d) *The skin by skin scraping:* the skin is shaved if necessary, cleaned with 70% ethanol and scraped with a sterile scalpel blade until liquid (exudate) emerges. The exudate is smeared on a slide and air-dried.

(e) *The nose:* by collecting intranasal secretions on a cotton swab. A cotton swab is rubbed firmly on the interior of the nose to obtain exudate from the mucosa, which is then smeared on a slide and air-dried.

6.4 **How is microscopy of aspirates and biopsies performed?**

Dried smears are fixed immediately with absolute (100%) methanol (methyl alcohol) for 2 minutes and stained with Giemsa at pH 7 - 7.2. The methanol must be stored in tightly closed bottles to prevent absorption of water.

Stain the films in Giemsa as described in Appendix 5.

Examine the Giemsa-stained smears using a 100X oil immersion objective. *Leishmania* amastigotes are very small round or oval organisms about 3 μm x 5 μm found inside or outside phagocytic cells (macrophages). Each amastigote contains a red-mauve nucleus, a smaller more deeply staining red-mauve kinetoplast, and pale blue cytoplasm. The particular diagnostic feature to look for in VL is the presence of nucleus and kinetoplast in these organisms.

In addition if local conditions allow the preparation of appropriate culture medium (as described in appendix 4) material from lymph node or bone marrow aspirates can be placed aseptically into culture. If cultures are not contaminated with bacteria or fungi free-swimming flagellated promastigote forms may be found when the cultures are examined by microscopy 7 - 14 days after they were inoculated with aspirates. Material from skin biopsies is much more difficult to culture. If this is to be attempted skin is swabbed first with 70% alcohol containing a few flakes of dissolved iodine and then swabbed again with 70% alcohol before the skin biopsy is taken aseptically.
6.5 **How is a serological diagnosis made?**

Useful serological tests are the formol gel test (aldehyde test), in the absence of any other, in tubes or on slides, the direct agglutination test (DAT), the indirect immunofluorescence test (IFAT) and the enzyme-linked immunosorbent assay (ELISA or Dot-ELISA). These tests are described fully in Appendices 6 (formol gel test), 7 (DAT test), 8 (IFAT) and 9 (ELISA).

The formol gel test and DAT (given a reliable standardised source of DAT antigen) can be performed outside laboratories in primary health care centres. The IFAT and ELISA tests require laboratory facilities and more sophisticated equipment, although rapid ELISA or dipstick tests are being developed for use in primary health care centres or in the home.

6.6 **How can infected dogs be treated?**

There is no proven way of obtaining a permanent cure in dogs. The following scheme of repetitive treatment is sometimes used in Europe to obtain temporary clinical improvement.

Pentavalent antimony: at 5 mg/kg/day for 14 - 30 days, repeated every 5 - 6 months.

In almost all cases the infection relapses after each course of treatment. This method of treatment may induce resistance of *Leishmania* parasites to pentavalent antimony and dogs may then act as a reservoir of infection for human disease.

*It is recommended therefore that drugs used to treat human infections must not be used to treat dogs. In this way the development of resistant parasites might be avoided.*

6.7 **How can infected dogs be prevented from infecting sandflies?**

At present there is no effective way that infected dogs can be prevented from infecting sandflies. Collars, soaps, shampoos and pyrethroids sprayed on dogs have not yet been proven to be effective.

6.8 **How can dogs be protected from being infected?**

There are no protection methods for dogs in endemic areas. Removal of the dog from the endemic area appears to be the only way to protect it from VL infection.
6.9 **What are the criteria for destroying an infected dog?**

Note that in some endemic areas of human VL canine VL is not known to occur and dogs are not therefore considered to be relevant as a reservoir of human infection (see appendix 2).

In many endemic areas governments have stated policy and laws which regulate the elimination of VL infected dogs.

In general all dogs proven to be parasitologically positive should be destroyed as they can act as a source of infection for sandflies and source of human disease.

In endemic areas of human VL it is often recommended that all serologically positive dogs are destroyed as they almost certainly carry active infections and may contribute to the spread of human disease.

In Europe dogs are sometimes treated repeatedly with pentavalent antimony but this is not considered appropriate if the same drug is used to treat human infections as treatment of dogs in this way can give rise to drug-resistant parasites (see section 6.6).

In many endemic areas the implementation of governmental policies and the principle that all parasitologically proven positive dogs should be destroyed is limited by financial resources and availability of reagents and equipment for the humane disposal of dogs (section 7).

6.10 **To whom and how should confirmed cases of canine VL be reported?**

Canine VL infections should be reported to appropriate local veterinary and public health authorities as this will provide important epidemiological information on the distribution and potential spread of the disease (see section 8).

6.11 **What follow-up action should the veterinary surgeon consider (to inform and protect the community)?**

Veterinary surgeons should contribute to the devising and distribution of publicity material (leaflets, posters, contributions to the media) to advise owners of infected dogs and their families of the epidemiology of VL, the prognosis of canine VL and potential risks to human health. Other dog owners in the infected area should also be made aware of the potential risk and consequences of canine VL.

Veterinary surgeons should also contribute to health education and community participation activities.
6.12 **What minimum or special equipment and services are required?**

For microscopical examination of stained smears: microscope with (50 X) or 100X oil immersion objective, microscope slides, staining trays, syringes (10 or 20 ml), needles, sodium chloride, disodium hydrogen phosphate, Giemsa stain.

Access is also required to the reagents and equipment for serological diagnosis of canine VL (see appendices 6 - 9).
7: VL AND PUBLIC HEALTH INTERVENTIONS

7.1 How can sandfly vectors be controlled?

Sandflies that rest inside buildings (endophilic vectors) can be controlled by spraying houses, chicken houses, stables, etc., with residual insecticides. Sandflies that rest outside houses (exophilic vectors) cannot be controlled in this way. (Appendix 12)

Repeated use of ultra-low volume application of insecticides to the entire community can reduce sandfly numbers but this is a method for use only in epidemics.

7.2 What personal protection methods can be used to prevent attack by sandflies?

Use of fine mesh mosquito nets or preferably nets impregnated with synthetic pyrethroids (the impregnation of bednets by insecticides allows the use of bigger meshes), (Appendix 3) provides good personal protection, especially in foci where humans are the sole reservoir.

Screening houses with fine mesh nets and/or curtains may reduce the density of sandflies indoors.

The establishment of settlements in areas of known high risk should be avoided.

7.3 How are epidemiological surveys for human cases conducted?

Passive case detection

Passive case detection depends on:

(a) an obligatory system of notification by which public health authorities are informed of each case (and records of all cases are assembled);

(b) a standardised diagnostic service (see sections 1, 2 and 3);

(c) a constant supply of drugs.

All diagnosed cases must be treated and should be followed up.
Active case detection

This is to discover undetected cases.

A search is made in the community for individuals with signs or symptoms of VL (Slide 38).

Blood samples are taken in the field for serological diagnosis. Individuals with a doubtful clinical picture may be referred for parasitological diagnosis.

Diagnostic methods that are applicable to active care detection are described in section 3. A positive leishmanin skin test is usually only found in patients who have recovered from infection and this can be used to help distinguish present and past cases.

All patients found to have clinical VL must be treated and should be followed up.

7.4 How are dogs systematically screened and controlled?

Passive case detection of canine leishmaniasis depends upon reports from veterinarians who should be required to report cases to a central authority (for example, the Ministry of Health or the organization responsible for the Control of Zoonotic Diseases).

Active case detection requires prior announcements in the community to inform them when and where to gather with their dogs for a free examination. More dogs can be surveyed in this way than examining dogs door-to-door. Active case detection can also be associated with rabies vaccination programmes (section 7.7).

The name and address of the owner and dog are recorded. Each dog is examined for clinical signs of canine VL, bled for serological diagnosis and samples taken for parasitological diagnosis (see section 6). Results of serological and parasitological tests should be communicated via civic authorities to owners, with recommendations to have all parasitologically positive dogs destroyed. In endemic areas of human VL it is recommended that all serologically positive dogs be destroyed as they almost certainly carry active infections and will contribute to the spread of human disease.

Parasitological tests are in general less sensitive than serology but even serology is likely to fail to detect some infected dogs, especially those in the early stages of VL.

7.5 What health education/publicity measures are required?

The first priority is to train the health professionals to perform effectively the functions required at each level of disease control (primary health care worker, physician, laboratory diagnostician, veterinary surgeon etc.).

Schools and other organizations (e.g. religious communities) should be used as entry
points to inform and motivate the community, and to explain the benefits of control of human (and canine) VL.

All health education should be accompanied by clinical support and early intervention.

All means of communication (posters, leaflets, radio, TV, slides, videos) should be exploited in health education.

7.6 **How should priorities be assigned to control measures?**

Intervention should be assessed in terms of cost and feasibility.

The first priority is the early treatment of passively detected human cases.

The second priority is the improvement of the detection, diagnosis and treatment of human cases especially in foci where humans are the sole reservoir.

The third priority is the training of all personnel involved in control.

In foci where the vector is known to be endophilic, and the number of cases is high, house-spraying with residual insecticides is an effective control measure and is the next priority.

In foci where domestic dogs are reservoir hosts, the efficacy of detecting and destroying infected dogs as the only or primary means of control is currently being questioned (see section 9).

Components of a control campaign against VL should be (a) coordinated and integrated (see section 8), (b) complete (not interrupted by failure of resources), and (c) sustained long enough to have an impact (usually several years for VL).

7.7 **How can control of VL be integrated with other disease control programmes?**

House-spraying with insecticides should be coordinated with other programmes aimed against arthropod-borne disease, e.g., against vectors of malaria, Chagas disease, Dengue, Japanese B encephalitis, etc.

Control of canine VL should be integrated with the control of rabies and hydatid disease.

7.8 **What special measures are required in response to epidemics?**

A local plan of action must be ready with the aim of halting epidemics. This plan should be updated annually in the light of new information. The plan must include provision for
gathering funds and allocating local, regional and national resources for prompt mass diagnosis, treatment and intervention. Prompt and effective treatment is of high priority in foci where humans are the sole reservoir in order to arrest spread of the epidemic. Measures for vector and reservoir controls should be implemented provided sufficient information can be obtained about the epidemiological cycle. Ultra low volume spraying (ULV) of insecticides is an additional measure for the control of epidemics. ULV provides an immediate, short-acting (a few hours only) but expensive reduction of high sandfly populations. For longer-term effect it needs to be repeated at short intervals (e.g. on consecutive days, followed by once or twice a week over several months). Public awareness should be improved by all means (press, handouts, radio, posters, television, educational programmes, etc.) and at all levels concerned. The population at risk should be given practical instructions on diagnosis and treatment.

7.9 How can the success of control programmes be evaluated?

Reduction in sandfly numbers may be difficult to quantify and by itself is not a sufficient means of evaluation of the success of control.

*The best criterion for evaluation is an annual reduction in the number of clinical cases in the human population.*

7.10 What minimum or special equipment and services are required for public health interventions (case management, vector and dog controls)?

Supply of drugs; record sheets; reagents and supplies for diagnosis; residual insecticides, equipment for individual protection; spraying equipment; provision for training, training materials, leaflets, posters and, if possible, audio visual means of public education; equipment and reagents for the humane killing of infected dogs.
8: VL AND THE MINISTRY OF HEALTH

8.1 What minimum or special infrastructures and personnel must be provided at primary, secondary and tertiary health care levels?

The first level which usually correspond to the health posts, dispensaries or primary health care centres, should be provided with a health worker. They are responsible for detection and notification of suspected cases.

The second level (health centres) should be provided with a physician, nurse, laboratory technician and a technical team for vector control and, if appropriate, animal reservoir control. They are responsible for confirmation of the clinically suspected cases and their treatment, and for vector and, if appropriate, reservoir control. They have to ensure the follow up of treated patients.

The third level (hospitals) should be responsible for the management of severe cases which need hospitalization. They are in charge of data collection and active surveillance.

The central level (National Health Ministry) will be responsible for:

- elaboration of health policies and guidelines for national strategies
- financial administration, cost-effectiveness analysis
- personnel administration
- provision of supplies: drugs, insecticides, reagents for diagnosis, spraying equipment
- training
- elaboration and distribution of training material
- collection, analysis and diffusion of data
- overall supervision and evaluation of the control programme and links to other control programmes
- epidemic control measures
- bilateral and multilateral cooperation
- links with research institutions

8.2 What minimum or special equipment and services must be provided for each activity and at each level?

Information on equipment and services required at each level is given in the appropriate sections of this report and in the relevant appendices.
8.3 **What health education material is required and how should it be disseminated?**

Any possibility of health education should be exploited and material diffused by all means of mass communication. Health education must be correctly targeted. People should get a clear perception of the advantages they can expect. Guidelines such as those described in this present volume should be sent to the appropriate level and be supported by training programmes.

Public awareness material for the community should be prepared at the central level.

8.4 **How can a supply of drugs, insecticides and essential reagents be assured?**

(a) First-line and back-up drugs required should be included in the list of Minimal Essential Drugs.

(b) Negotiation of a long-term competitive contract for insecticide supplies would be beneficial.

(c) A separate budget allocation for drugs and insecticides for VL control should be supported, especially to avoid interruption of interventions.

(d) Adequate storage for drugs and insecticide should be ensured.

(e) When feasible, and especially in case of an epidemic, a local task force should be set up to coordinate and supervise the distribution and use of reagents, drugs and insecticides.

8.5 **What back-up resources are required to respond to epidemics?**

(a) Elaboration of a strategic plan and designation of a task force.

(b) Stocks of drugs, reagents, insecticides and equipment.

(c) Availability of reserved personnel to implement the emergency plan of action.

(d) Logistical support including transport.

(e) Prompt involvement of other infrastructures: when there is no specific leishmaniasis unit, existing health infrastructures should be used such as those of malaria.

(f) Special forms for reporting cases

(g) Material for health education and training.
8.6 **What minimal communication/disease notification network is necessary?**

(a) The minimum essential communication should be by voice (telephone or radio) at all levels.

(b) Additional written communication is required at all levels (courier, fax).

(c) At the central level, computer availability is necessary for the collection and analysis of data (and for e-mail).

8.7 **What monitoring of the control activities are required?**

(a) Recording changes in the numbers of cases (per month and year) possibly as changes in incidence if population fluctuations are marked but known from census data.

(b) External quality control for diagnosis.

(c) Follow-up of treated patients.

(d) Monitoring of utilisation of resources.

(e) Monitoring availability of personnel.

8.8 **What are the benefits of disease prevention and control?**

- Reduced cost for treatment or hospitalization
- Maintenance of family income
- Sustained labour and production
- Reduction of morbidity and mortality
- Continued education of children
- No social consequences
- No destruction of the community
- Sustained income from tourism
- More cost-effective health care
9: VL AND THE RESEARCH SCIENTIST

9.1 Is more research on VL needed (applied, basic)?

Further research is needed both at the basic and the applied levels.

9.2 If so, what are the recommended research objectives?

The priority research needs in VL research are:

(a) The development of new therapies (for example drugs, or immunological therapies) for human VL, preferably drugs that can be given orally in single or few doses at an affordable price and with no significant side effects.

(b) The production of simple, specific, fast, inexpensive, and highly sensitive, antibody detection test for the diagnosis of VL, preferably for field use.

(c) The production of effective vaccines for VL.

(d) The identification of the most sustainable vector control strategy (evaluation of insecticide impregnated bednets).

(e) Cost-effective analysis of VL control strategies.

(f) Methods for the improved diagnosis and treatment of PKDL.

(g) Identification and quantification of the risk factors involved in the acquisition of VL.

(h) Evaluation of the impact of the elimination of dogs (parasitologically and/or serologically positive) on VL transmission.

(i) The development of vector population density indicators for use in endemic areas and in epidemics.

(j) An understanding of the mechanisms of drug resistance in *Leishmania*.

(k) An understanding of the mechanisms of epidemics.

9.3 What is the order of priority of the research objectives?

The order of priority varies according to the needs of each country. Each country has to establish an order of research priorities to best fit the needs of that country.
Overall the research priorities and those which will have greatest impact on the control of VL are: the development of new drugs, new diagnostics and new vaccines together with studies to analyse the most cost-effective interventions. Overall research priorities are therefore considered to be as listed in 9.2 above.

9.4 **What are the sources of funding for such research?**

Examples of known sources of funding are:

- Individual governments and government agencies.

- Commission of the European Communities  
  Rue de la Loi 200  
  B-1049 Brussels  
  BELGIUM

- International Development Research Centre  
  P. O. Box 8500  
  Ottawa, K1G 3H9  
  CANADA

- National Institutes of Health  
  Bethesda, Maryland 20892-0425  
  U. S. A.

- Overseas Development Administration  
  94 Victoria Street  
  London, SW1E 7JL, U.K.

- Special Programme for Research and Training in Tropical Diseases (TDR)  
  World Health Organization  
  CH-1211 Geneva 27  
  SWITZERLAND

- The Wellcome Trust  
  183 Euston Road  
  London, NW1 2BE, U.K.

There are many other potential sources of finance, including local charities, commercial sponsors, and other non-governmental international organizations, which should be explored to obtain funding for priority research.
APPENDICES

APPENDIX 1  Geographical distribution of VL
APPENDIX 2  Regional differences in the epidemiology of VL and their effect on disease control methods/strategies
APPENDIX 3  Insecticide impregnation of mosquito nets
APPENDIX 4  Culture of *Leishmania* from aspirate or biopsy samples
APPENDIX 5  Giemsa staining and haematology
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APPENDIX 10  Leishmanin skin test (Montenegro)
APPENDIX 11  Drug supplies, costs and import restrictions
APPENDIX 12  Insecticides and their use
APPENDIX 1

Geographical distribution of VL

61 countries

1.1) Central and South America (11)

Argentina, Bolivia, Brazil, Colombia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Venezuela.

1.2) Europe (15)

Albania, Azerbaidjan, Cyprus, France, Georgia, Greece, Italy, Kazakhstan, Malta, Portugal, Spain, Tajikistan, Turkey, Turkmenistan, Uzbekistan.

1.3) Southwestern Asia (10)

Afghanistan, Iraq, Islamic Republic of Iran, Israel, Jordan, Lebanon, Oman, Saudi Arabia, Syrian Arab Republic and Yemen.

1.4) Africa (20)


1.5) Asia (5)

Bangladesh, China, India, Nepal and Pakistan
## APPENDIX 2.1

### ANTHROPOONOTIC VISCERAL LEISHMANIASIS

<table>
<thead>
<tr>
<th>CLINICAL DISEASE</th>
<th>INDIAN SUBCONTINENT</th>
<th>EAST AFRICA</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Incubation</td>
<td>2 weeks to 6 months</td>
<td>weeks to months</td>
</tr>
<tr>
<td>• Age distribution</td>
<td>children, teenagers, young adults (70% 5-30 years)</td>
<td>children, teenagers, young adults (75% 5-30 years)</td>
</tr>
<tr>
<td>• Sex</td>
<td>$\sigma &gt; \varphi$</td>
<td>$\sigma &gt; \varphi$</td>
</tr>
<tr>
<td>• PKDL</td>
<td>frequent</td>
<td>frequent</td>
</tr>
<tr>
<td>• Outbreaks</td>
<td>frequent, high mortality rate</td>
<td>frequent, high mortality rate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LEISHMANIA Species:</th>
<th>$L., donovani$</th>
<th>$L., donovani$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locations:</td>
<td>SRE/blood/normal skin</td>
<td>SRE/blood/nasal mucosae/normal skin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VECTOR Species:</th>
<th>$P., argentipes$</th>
<th>$P., orientalis/P., martini/P., celiae$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locations:</td>
<td>intra and peridomiciliary (houses &amp; cowsheds)</td>
<td>acacia forests + termite hills</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RESERVOIR</th>
<th>man</th>
<th>man</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOTOPE</td>
<td>rural (villages)</td>
<td>acacia forest houses near termite hills</td>
</tr>
</tbody>
</table>
# APPENDIX 2.2

## ZOONOTIC VISCERAL LEISHMANIASIS

<table>
<thead>
<tr>
<th></th>
<th>SOUTHERN EUROPE</th>
<th>MAGHREB/EASTERN MEDITERRANEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLINICAL DISEASE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>· Age distribution</td>
<td>&gt; 50% adults</td>
<td>95% children &lt; 5 years old</td>
</tr>
<tr>
<td>· Sex</td>
<td>♂ &gt; ♀</td>
<td>♂ &gt; ♀</td>
</tr>
<tr>
<td>· Outbreaks</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td><strong>LEISHMANIA Species:</strong></td>
<td><em>L. infantum</em></td>
<td><em>L. infantum</em></td>
</tr>
<tr>
<td><strong>VECTORS Species:</strong></td>
<td><em>P. ariasi</em></td>
<td><em>P. langeroni</em></td>
</tr>
<tr>
<td></td>
<td><em>P. perniciosus</em></td>
<td><em>P. longiductus</em></td>
</tr>
<tr>
<td></td>
<td><em>P. perfiliewi</em></td>
<td><em>P. major</em></td>
</tr>
<tr>
<td></td>
<td><em>P. neglectus</em></td>
<td><em>P. syriacus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. smirnovi</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. tobbi</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. chinensis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. perniciosus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. longicuspis</em></td>
</tr>
<tr>
<td><strong>RESERVOIRS Species:</strong></td>
<td>dog/fox</td>
<td>dog/fox/jackal/black rat?</td>
</tr>
<tr>
<td><strong>BIOTOPES</strong></td>
<td>small villages (rural) perurban</td>
<td>small villages (rural)</td>
</tr>
</tbody>
</table>
### APPENDIX 2.3

**ZOONOTIC VISCERAL LEISHMANIASIS**

<table>
<thead>
<tr>
<th></th>
<th>CHINA</th>
<th>CENTRAL/SOUTH AMERICA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLINICAL DISEASE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Age distribution</td>
<td>children 95% &lt; 10 years old</td>
<td>children 70% &lt; 5 years old</td>
</tr>
<tr>
<td>• Sex</td>
<td>$\sigma &gt; \varphi$</td>
<td>$\sigma &gt; \varphi$</td>
</tr>
<tr>
<td>• Outbreaks</td>
<td>no</td>
<td>small outbreaks in suburban areas</td>
</tr>
<tr>
<td><strong>LEISHMANIA Species:</strong></td>
<td><em>L. infantum</em></td>
<td><em>L. infantum (L. chagasi)</em></td>
</tr>
<tr>
<td><strong>VECTOR Species:</strong></td>
<td><em>P. chinensis</em></td>
<td><em>Lu. longipalpis (peridomestic)</em></td>
</tr>
<tr>
<td></td>
<td><em>P. longiductus (peridomestic)</em></td>
<td><em>Lu. evansi</em></td>
</tr>
<tr>
<td></td>
<td><em>P. major wui</em></td>
<td></td>
</tr>
<tr>
<td><strong>RESERVOIR Species:</strong></td>
<td>dog/raccoon dog</td>
<td>dog/fox/marsupial</td>
</tr>
<tr>
<td><strong>BIOTOPE</strong></td>
<td>small villages (rural)</td>
<td>small villages (rural)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>suburban (poor sanitary conditions)</td>
</tr>
</tbody>
</table>
Figures A and B

Shapes of two commercially made mosquito nets for beds. Rectangular net (A) has four points for tying, and the conical net (B) has only one. Sections to be measured to calculate the area of each are also indicated.
APPENDIX 3

Insecticide impregnation of bednets*

The insecticide is always mixed with water and the net is soaked with the appropriate quantity of solution to give the required dosage after drying of the net.

3.1 Calculation of bednet area to be treated

There are a large variety of nets, home-made or industrialized, heavy/light and strong/fragile materials (cotton, nylon and polyester or other textiles) of different mesh sizes (number of holes/square inch), different weights (40, 75, 100 denier), breaking strength, colors, sizes (from 8 m² to 25 m²) and shapes (conical, rectangular, half-cubic, with or without slit-doors or sheeting borders).

However, two main types of bednets are commercialized. Rectangular nets (Fig A) have four points for tying and the conical net (Fig B) has only one. Sections to be measured to calculate the area of each type are indicated on Figures A and B.

3.1.1 Rectangular bednet (Fig A): determine the area of an end (S1 = height x width, a x c), a side (S2 = height x length, a x b) and the upper part (S3 = width x length, b x c). The following formula should be used to estimate the total area to be treated: \( S = 2 \times (S1 + S2) + S3 \).

3.1.2 Conical bednet (Fig B): is usually pleated at the top and is really a cylinder rather than a cone. Thus, measure the height (d) from the bottom to the top and the circumference at the wide end (e). The total area to be treated is: \( S = e \times d \).

3.2 The amount of technical grade pyrethroid (active ingredient, a.i.) needed to treat a net (target deposit density):

- permethrin (EC 25 %): the treatment rate is 500mg a.i./m²
- deltamethrin (EC 2.5 %): 25 mg a.i./m²
- cypermethrin (EC 10 %): 100 mg a.i./m²
- lambacyhalothrin (EC 2.5%): 25 mg a.i./m²
3.3 Calculation of the amount of pyrethroid needed to treat a net

After determining the amount of technical grade needed (target deposit density), use the following formula to calculate the amount of emulsifiable concentrate (EC) needed:

\[
\text{target deposit density (g/m²) x area of fabric (m²) x 100} \times \% \ of \ active \ ingredient \ in \ the \ EC
\]

An EC of the insecticide is preferred over a water-dispersable powder formulation since it provides better adhesion to the net material and does not leave a powdery residue.

3.4 Impregnation

- In a non-absorbent container, such as a plastic bag or basin, determine the amount of water necessary to saturate but not run off the net. A cotton net absorbs a considerable amount of water while a nylon net is not absorbent and thus will require considerably less water for treatment. Cotton nets usually require 30 ml of water/m² of net; nylon nets require 30 ml of water/2 m²;

- Wear gloves and mix thoroughly the amount of pyrethroid, as calculated, with the previously determined amount of water and soak the bednet (be sure the mixture totally covers the fibres) in the liquid (insecticide emulsion) until it is fully impregnated;

- Remove the excess liquid by rubbing and squeezing the net in order to obtain a uniform distribution of the insecticide over the whole net. The excess solution must be collected;

- Let the bednet dry in the shade in a clean area on a non-absorbent surface such as polythene bags in a horizontal position to avoid the impregnation liquid from flowing away. It can also be dried indoors on a bare mattress. Do not hang to dry;

- When the bednet is dry, it is ready for use. It can be also be stored for some days in a kraft paper wrapping for future use.

3.5 Precautions

- Wear gloves to avoid any contact with the skin, especially with the mucosae. In case of accidental contact with the insecticide, rinse abundantly with water and any burning sensation will disappear in few hours with no sequelae.

- Keep out of reach of young children (to prevent contact with the mouth).
- Do not wash the bednet during the transmission season or if it is washed reimpregnate it after each washing. Although part of the insecticide remains, it is safer to reimpregnate it systematically every time that it is washed.

- Do not discard any remaining solution of diluted insecticide in ponds, rivers or any other breeding sites for fishes as pyrethroids are toxic for cold-blooded animals.

- All the bednets must be fire-resistant.

* The above text is adapted from:
APPENDIX 4

Culture of Leishmania from aspirate or biopsy samples

Wherever possible use a blood agar based culture medium, as for initial isolation of Leishmania, they are the most reliable. In VL, isolations are usually made from aspirates of bone marrow, spleen or lymph gland. Aspirated material is taken aseptically, and into an anticoagulant in the case of bone marrow and spleen, and inoculated into one of the culture media described below. Do not inoculate large volumes of marrow or splenic aspirates as these contain substances inhibitory to the growth of Leishmania promastigotes. Using aseptic precautions inoculate two or at most three drops of splenic or marrow aspirate into each culture tube. Inoculate several tubes, and incubate at 25°C or below. Examine under a microscope a small drop of culture medium taken aseptically from each tube every 48 hrs for the growth of promastigotes. The majority of cultures destined to become positive will do so within 7 - 10 days. Those still negative at 10 days should be blind passaged into fresh culture medium and then examined as before. Discard any culture still negative after 20 days.

Culture media recipes

Proline Balanced Salts Solution (PBSS)

PBSS although not a complete culture medium in itself is a component of most of the media described below, and a stock of this should always be available. Its composition is as follows:

KCL  
0.4 gm

NA₂HPO₄. 12H₂O  
0.06 gm

KH₂PO₄  
0.06 gm

CaCl₂. 2H₂O  
0.185 gm

MgSO₄. 7H₂O  
0.1 gm

MgCl₂. 6H₂O  
0.1 gm

NaCl  
8.0 gm

L-proline  
1.0 gm

Phenol red  
0.001 gm

Distilled water  
1000 ml
Dissolve the ingredients one at a time in approx 70 ml of distilled water. Adjust the pH to 7.2 with a few crystals of Tris (Tris[hydroxymethyl]-aminomethane), make up the volume to 1000 ml, dispense into convenient screw-capped bottles, and sterilise by autoclaving at 121°C for 15 min. Store preferably at 4°C, although it will keep for several months at room temperature.

"Sloppy" Evans Medium

One of the most successful culture media for isolating Leishmania from patients with VL is "Sloppy Evans" medium.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBSS (see above)</td>
<td>85 ml</td>
</tr>
<tr>
<td>Bacteriological peptone</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>0.03 gm</td>
</tr>
<tr>
<td>Defibrinated rabbit blood</td>
<td>15 ml</td>
</tr>
<tr>
<td>Agar (plain non-nutrient)</td>
<td>0.3 gm</td>
</tr>
</tbody>
</table>

To prepare, mix the ingredients (omit the defibrinated rabbit blood) in a screw-topped bottle. Sterilise by autoclaving at 121°C for 15 min, cool to about 50°C, add the blood then a solution of gentamycin to give a final concentration of 50 µg/ml of medium (i.e. 5 mg gentamycin in 100 ml of complete medium as above). Mix well and dispense while still molten into suitable sterile tubes or bottles (3 ml into a bijou bottle of 7 ml capacity is ideal). Inoculate the material aspirated from the patient deeply into the sloppy agar.

Biphasic blood agar media

(a) NNN medium

Solid phase: heat 1.4 gm agar (plain non-nutrient), 0.6 gm NaCl and 90 ml of distilled water together in a flask; keep the contents well mixed until the agar melts. Transfer the molten agar to a screw-topped bottle and sterilize by autoclaving at 121°C for 15 min, cool to about 50°C, add 10 ml of defibrinated rabbit blood to which 5 mg gentamycin has been added, then dispense whilst molten into sterile culture tubes or bottles. Place the tubes or bottles in a sloped position until the agar has set, then transfer to a refrigerator.

Liquid phase: classically this consists of the water that condenses at the bottom of the slopes, but in practice most workers add additional liquid phase such as PBSS or even sterile distilled water. If additional liquid phase is added do not add more than 5 drops to a slope made in a 7 ml bijou bottle. Inoculate the material from the patient into the liquid portion of the medium.

*Rabbit blood collected aseptically and agitated with sterile glass beads to remove fibrin.*
(b) USMARU medium (DIFCO blood agar medium)

Solid phase: 4 gm 'Bacto' blood agar base and 100 ml distilled water. Preparation as for NNN medium, including the addition of defibrinated rabbit blood and gentamycin. The liquid phase is also the same as for NNN.

Notes

Use of bloods other than rabbit blood in biphasic media

If you cannot obtain rabbit blood it is worthwhile trying other bloods that may be available. Sheep, horse or human bloods have all been used with some degree of success. Use them either defibrinated or with an anticoagulant, but always heat inactivate (56°C for 30 min) before use and if possible increase the agar-agar concentration in the medium to 2%.

Storage. Store prepared tubes at 4°C, these media are best used within one week of adding the blood, and ideally should be discarded after three weeks storage at 4°C. The medium without the blood may be stored for several months at ambient temperature.
APPENDIX 5

Giemsa staining and haematology

Giemsa staining

Reagents

Giemsa stain solution (e.g. BDH/Merck Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, R66; Product 35086). The Giemsa stain may have variable batch quality. Each new batch should be checked against a known organism before routine use.

Phosphate-buffered distilled water, pH 7.2.

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 0.7 \text{ gm} \\
\text{Na}_2\text{HPO}_4 & \quad 1.0 \text{ gm} \\
\text{Distilled water} & \quad 1.0 \text{ litre}
\end{align*}
\]

Absolute methanol (Analar). This must be stored in a tightly stoppered bottle to prevent absorption of water.

Method

1. Prepare thin film as for routine haematology. Ensure that the film has a good ‘tail’ and does not reach the edges of the slide laterally.

2. Allow the film to dry in air and fix with methanol for one minute.

3. Tip off excess methanol and place face down on a slide staining tray.

4. Using a 20 ml syringe and a blunt needle, dilute the stock Giemsa 1:10 with buffered distilled water. Mix well and expel air.

5. Infiltrate the stain, using the syringe and needle, under the slide, taking care not to trap large air bubbles. Stain for 25 - 30 minutes.

6. At end of staining time, rinse slides briefly with tap water and allow to drain dry in a vertical position. Possible parasites should be examined in more detail using the oil immersion lens. A 50x or 63x oil immersion objective is invaluable for preliminary examination: at least 1000 fields should be examined by a trained microscopist.
Notes:

The syringe method for dilution of Giemsa (point 4 above) is strongly recommended, as once the stain is diluted with water precipitation of the stain begins which is hastened by exposure to air. This dilution must, therefore, be prepared immediately prior to staining; stock diluted stain should not be made. Staining face-downwards in a slide tray also reduces precipitation, and any that does develop falls away from the smear. Cleanly stained smears are very important when searching for small intracellular parasites.

The buffered water at pH 7.2 must be used for the dilution of stain for blood parasites. It is only at this alkaline pH that proper differentiation of parasite nuclear and cytoplasmic material takes place.

Packed cell volume (PCV) determination (haematocrit)

The packed cell volume (PCV) is the percentage of blood volume taken up by red cells. A low PCV is indicative of anaemia. To determine the PCV a heparinised microhaematocrit capillary tube is filled directly with capillary blood (e.g. from lancet puncture of the finger or ear) or filled with venous blood collected with anticoagulant. Tubes are sealed at one end with Plasticine or by heat. Use capillary action to fill the tubes leaving 10 - 15 mm unfilled at the end of the tube to be sealed. The tube is centrifuged in an haematocrit centrifuge (at 12,000 g) with the sealed end of the tube against the rubber outer edge of the centrifuge plate. Centrifuge for 5 minutes and read the PCV in a microhaematocrit reader by putting the base of the red cell layer on the zero and the top of the plasma layer on the 100 lines. The silver line position is adjusted to touch the red cell/white cell/platelet interface and the PCV volume is read from the scale.

The PCV (haematocrit) is the ratio of packed red cells to the volume of the blood.

White cell count

White cell diluting fluid:

- Glacial acetic acid 2 ml
- 1 % methylene blue 1 - 2 drops
- Distilled water to 98 ml

The red cells are lysed and a stain is used for the white cells. To lyse the cells 20 μl of blood is added to 0.38 ml of white cell diluting fluid (1 in 20): mix thoroughly. A
haemacytometer counting chamber is filled and left to stand in a humid chamber for
two minutes. All the white cells in an area of 4 mm$^2$ are counted.

The white cell count

\[ \text{no. of cells counted} \times \text{dilution} \times 10^6 = \text{the no. counted} \times 50 \times 10^9/l \]

volume counted (0.4)

Normal white cell counts (WBC) in different age groups are:

<table>
<thead>
<tr>
<th>Age group</th>
<th>Normal WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants at 1 year</td>
<td>6-18 x 10$^9$/l</td>
</tr>
<tr>
<td>Children 1 to 10 years</td>
<td>5-14 x 10$^9$/l</td>
</tr>
<tr>
<td>Adults</td>
<td>4-11 x 10$^9$/l</td>
</tr>
</tbody>
</table>

**Determination of haemoglobin (cyanmethaemoglobin method)**

Solution for dilution of blood (Drabkin's solution, pH 7.0 - 7.4)

- Potassium ferricyanide: 200 mg
- Potassium cyanide: 50 mg
- Potassium dihydrogen orthophosphate: 140 mg
- Nonidet P40: 1 ml
- Distilled water: to 1 l

Store at room temperature in the dark (do not freeze). *This solution is highly toxic.*

This solution can be prepared by dissolving standard tablets of components or by
diluting an ampoule of commercially available concentrate.

Add 20 $\mu$l of blood collected with anticoagulant to 4 ml of reagent, mix well and leave
at room temperature for three minutes. The absorbance is read on a colorimeter at
540 nm against a blank of the reagent solution alone. The absorbance of a reference
standard of cyanmethaemoglobin is also measured separately against a reagent blank.
Haemoglobin concentration (gm/l) is calculated by:

\[
\text{Absorbance of test sample} \times \text{Concentration of standard} \times \text{dilution factor}
\]

Absorbance of standard \hspace{1cm} 100

Normal ranges are:

<table>
<thead>
<tr>
<th>Age group</th>
<th>Normal ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children 1 to 6 years</td>
<td>110 - 140 gm/l</td>
</tr>
<tr>
<td>Adult males</td>
<td>130 - 180 gm/l</td>
</tr>
<tr>
<td>Adult females</td>
<td>115 - 165 gm/l</td>
</tr>
</tbody>
</table>

Total serum protein

Biuret reagent:

- Copper sulphate (5 H₂O) \hspace{1cm} 3 gm
- Potassium sodium tartrate \hspace{1cm} 9 gm
- Potassium iodide \hspace{1cm} 5 gm
- NaOH \hspace{1cm} 24 gm
- Distilled water \hspace{1cm} up to 1 l

Alkaline tartrate reagent:

- Potassium sodium tartrate \hspace{1cm} 9 gm
- Potassium iodide \hspace{1cm} 5 gm
- NaOH \hspace{1cm} 24 gm
- Distilled water \hspace{1cm} up to 1 l

Add 0.1 ml of serum to 5 ml of biuret reagent.
Add 0.1 ml of serum to 5 ml of alkaline tartrate solution.
Allow both tubes to stand for 30 min at room temperature.

Blank a spectrophotometer with a 540 nm wavelength filter, using 5 ml of biuret reagent mixed with 0.1 ml of distilled water.

Read the absorbance of the patient’s serum in biuret reagent and subtract the absorbance in alkaline tartrate solution.

The total protein in the patient’s sample can be read from a calibration curve prepared from 5 dilutions of a protein standard solution or a control serum containing a known quantity of protein. Lyophilised standards containing known quantities of total protein are available commercially.
APPENDIX 6

Formol-gel test

Add two drops of concentrated formalin solution (40 % w/v) to approximately 1 ml of serum. In a positive test the serum turns white and solidifies into a gel. In a negative test the serum remains unchanged or whitening and gelling only occur after some delay (30 minutes). Solidification of the serum without whitening is regarded as a negative test for VL.

The formol-gel test may also be performed with smaller quantities of serum on a slide. Mix one drop of serum and a small drop of concentrated formalin solution together and look for whitening and solidification of the serum into a gel.
APPENDIX 7

Direct Agglutination Test

Organism:  *In vitro* cultivated promastigotes of (a local strain of) *L. donovani*

*L. infantum/L. chagasi*

Reagents:

- **Locke’s solution:**
  - Glucose: 0.25 % (w/v)
  - Sodium chloride: 0.9 % (w/v)
  - Potassium chloride: 0.04 % (w/v)
  - Calcium chloride: 0.02 % (w/v)
  - Sodium bicarbonate: 0.02 % (w/v)

- **Citrate saline:**
  - Sodium chloride: 8.77 gm
  - Distilled water: to 1000 ml
  - Adjust pH to 7.4 by addition of 0.056 M tri-sodium citrate (16.46 gm/1000 ml).

- **Diluent:**
  - Use citrate saline pH 7.4 containing 1% (v/v) heat-inactivated foetal calf serum *
  - and 0.1 M 2-mercaptoethanol (0.2 M for dogs).

*Note* * Foetal calf serum may be replaced in the diluent by 0.2 % gelatin. Add the gelatin to the citrate saline to give a final concentration of 0.2 % (w/v), heat at 56°C for 10 min. to dissolve the gelatin, leave to cool at room temperature, then add the 2-mercaptoethanol.
Antigen preparation:

1. Harvest promastigotes by centrifugation at 4000 g for 10 min at 4°C.

2. Wash (x5) by resuspension in cold Locke's solution and centrifugation at 3200 g - 10 min at 4°C.

3. Prepare solution of trypsin (0.4% w/v Difco 1:250 trypsin) in Locke's solution, adjust pH to 7.7.

4. Add trypsin solution to the packed promastigotes in the proportion of one vol. of packed promastigotes to 20 vol. of trypsin solution.

5. Mix well to resuspend the promastigotes, then incubate at 37°C for 45 min.

6. Centrifuge the suspension (3200 g - 10 min), then wash (x5) as in (2).

7. Resuspend the pellet in cold Locke's solution to a concentration of approximately 2 x 10⁶ cells/ml.

8. Add an equal volume of 2% formaldehyde in cold Locke's solution. Leave at 4°C overnight.

9. Centrifuge at 3200 g - 10 min at 4°C. Wash pellet in cold citrate saline. Resuspend to same concentration as in (8).

10. Add Coomassie Blue to a final concentration of 0.1% (w/v). Leave for 90 min. stirring at a moderate speed on a magnetic stirrer.

11. Centrifuge (3200 g - 10 min.) and wash pellet (x2) in citrate saline.

12. Resuspend in citrate saline containing 0.4% formaldehyde to the same volume as (10).

13. Store at 4°C, protect from the light. DO NOT FREEZE.

Procedure for performing the DAT test

1. Use microtitre plates "V"-shaped wells not "U"-shaped. Prepare the microtitre plates: Number the plate, fill in the corresponding form: plate number, date and sample code.

2. Dilute the serum to be tested 1/100 with the citrate-saline/FCS/2-mercaptoethanol diluent. Incubate at 37°C for 30 min.
3. For a 12-row microtitre plate pipette 50 μl of diluent to all wells except no 2.

4. Into well 2 pipette 100 μl of the 1/100 dilution of the serum under test (see 1).

5. Transfer 50 μl from well 2 to well 3, mix then transfer 50 μl from well 3 to well 4. Continue this operation across the plate, discarding the 50 μl taken from well 12 at the end.

6. Positive and negative control sera should be systematically incorporated in separate wells.

7. Shake the DAT antigen gently to resuspend the organisms, then pipette 50 μl into well 1 (no serum control). Next pipette 50 μl antigen to well 12, 50 μl of antigen to well 11 and so on until all wells have received antigen.

8. Cover the plate with a lid or plastic film, tilt the plate gently clockwise and anti-clockwise for 60 seconds and incubate overnight at room temperature in a horizontal position away from bumps hazard. Be careful to avoid spillage from one well into another.

Reading the test. Place microtitre plate on a plain sheet of white paper or else on a light box and view the plate from above. Two independent readers should read the test.

Figure:

NEGATIVE : ◯ dark blue dot, of a size identical to the size of the antigen control dot.

POSITIVE : from film to dot > antigen control dot

100 % DA titer end titre
End point. This is taken as the last well where agglutination is seen, i.e. the well before a clear, sharp-edged blue spot "button" in the bottom of the well, like that seen in the serum-free control well (well 1) is observed.

Titers of $\geq 1/3200$ are usually considered as being positive for human VL (lower titers are sometimes used for canine VL).

The microtitre plates can be reused after reading the test on condition they are thoroughly cleaned with 0.25% sodium dodecyl sulphate (SDS), and sufficiently rinsed with distilled water and air dried. It is better to use new plates whenever possible.
APPENDIX 8

Immunofluorescent antibody test (IFAT)

Reagents

*Phosphate buffered saline (PBS), pH 7.2*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 gm</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>2.88 gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>up to 1 l</td>
</tr>
</tbody>
</table>

(Can be made up at a concentration of 10 x the above recipe for better long-term storage.)

*PBS/0 05 % Tween (PBS/T)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS solution</td>
<td>99.95 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

*PBS/T/2 % milk powder (PBS/T/M)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS/T solution</td>
<td>100 ml</td>
</tr>
<tr>
<td>Dried skinned milk (low fat)</td>
<td>2.00 gm</td>
</tr>
</tbody>
</table>

*PBS/10 % glycerol (v/v)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS solution</td>
<td>90.00 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10.00 ml</td>
</tr>
</tbody>
</table>

Cultured *L. donovani, L. infantum* or *L. chagasi* promastigotes are washed (x 3) with sterile phosphate buffered saline (PBS) by centrifugation and re-suspension, preferably at 4°C. The final pellet is re-suspended in PBS at 5 x 10⁸ promastigotes/ml and 5 μl volumes of cell suspension are immediately dispensed into each well of the IFAT microscope slides (e.g. from Henley, Essex, UK) and the slides are allowed to dry in air. The slides are wrapped in tissue paper, placed in plastic bags and stored at -20°C. Slides are retrieved from storage by allowing them to come to room temperature in a desiccator.
Serum samples are diluted in phosphate buffered saline/0.05 % tween/2 % milk (PBS/T/M). Serum sample dilutions, beginning with a dilution of 1:50 are added into each well and the slides are incubated for 30 min at room temperature in a humid chamber. After incubation the slides are washed (x 3) for 10 min with phosphate buffered saline/0.05 % tween (PBS/T). 5 μl of a 1:50 dilution of anti-dog immunoglobulin fluorescein isothiocyanate conjugate (FITC) in PBST/M/1:10,000 Evans blue is added to each well and the slides are incubated for 30 min at 37°C in a humid chamber. After incubation, the slides are washed (x 5) in PBS and allowed to air-dry in the dark. Each slide is mounted in PBS/glycerol and observed at a x 1000 magnification under a fluorescent microscope in a dark room.
APPENDIX 9

ELISA for anti-\textit{Leishmania} antibodies

\textbf{Reagents}

\textit{Coating Buffer (CB)}

\begin{itemize}
\item \textbf{Na}_2\text{CO}_3 \quad 1.59 \text{ gm}
\item \textbf{NaHCO}_3 \quad 2.93 \text{ gm}
\item \textbf{NaN}_3 \quad 0.2 \text{ gm}
\item Distilled water \quad \text{up to 1 l}
\end{itemize}

(Can be made up at a concentration of 10 x for more convenient storage.)

\textit{CB/2 \% milk powder}

\begin{itemize}
\item CB \quad 100 \text{ ml}
\item Dried skimmed milk (low fat) \quad 2.00 \text{ gm}
\end{itemize}

\textit{Phosphate buffered saline (PBS), pH 7.2}

\begin{itemize}
\item \textbf{NaCl} \quad 8 \text{ gm}
\item \textbf{KH}_2\text{PO}_4 \quad 0.2 \text{ gm}
\item \textbf{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \quad 2.88 \text{ gm}
\item \textbf{KCl} \quad 0.2 \text{ gm}
\item Distilled water \quad \text{up to 1 l}
\end{itemize}

(Can be made up at a concentration of 10 x the above recipe for better long-term storage.)
**PBS/0.05 % Tween (PBS/T)**

- PBS solution: 99.95 ml
- Tween 20: 0.05 ml

**PBS/T/2 % milk powder (PBS/T/M)**

- PBST solution: 100 ml
- Dried skimmed milk (low fat): 2.00 gm

**Phosphate citrate buffer, pH 5.5**

**Solution A**
- Citric acid: 2.1 gm
- Distilled water: to 100 ml

**Solution B**
- Na₂HPO₄ . 12H₂O: 3.5 gm
- Distilled water: to 100 ml

Add 48.5 ml of solution A to 51.5 ml of solution B.

**Substrate solution (for peroxidase HRP conjugate)**

- O-phenylenediamine hydrochloride (OPD): 0.040 gm
- Phosphate citrate buffer, pH 5.5: 100 ml
- Hydrogen peroxide (3 %): 30 μl

**Preparation of ELISA antigen**

*L. donovani, L. infantum* or *L. chagasi* promastigotes are cultured in liquid medium and log-phase promastigotes harvested at a concentration of approximately 1 x 10⁶ cells/ml. The cell pellet is washed (x 3) preferably at 4°C, with sterile phosphate buffered saline (PBS) and frozen at -20°C. The frozen pellet is thawed to room temperature and re-suspended in sterile distilled water at 1:40 (v/v). The cells are disrupted by rapidly freezing in liquid nitrogen and thawing in water at 37°C (x 3) and if possible followed by ultrasonication for 15 seconds (x 5). The disrupted cells are centrifuged at high speed (e.g. 10,000 g) for 15 minutes at 4°C and the supernatant stored frozen at -20°C for use as the ELISA antigen.
Coating of ELISA wells

Polystyrene microtitre plates or similar plates are used for ELISA but they must be plates that are specifically recommended for ELISA. The antigen dilution used for coating the wells is determined by a checker-board experiment using different antigen dilutions against standard positive and negative serum controls and several different test dilutions of the conjugate. Antigen dilutions are made in 0.05 M carbonate coating buffer, pH 9.6. To coat the wells 100 μl of diluted antigen is pipetted into each well and left at 4°C overnight. The wells are then washed (x 3) with PBS and blocked with coating buffer/2 % milk powder for one hour at 37°C and washed again (x 3) with PBS.

Performing the ELISA

Sera are diluted 1:200 in PBS/T/M. Further dilutions are made, e.g. doubling dilutions if serum titres are required. 100 μl of serum dilutions are added to each antigen coated well of a 96 well ELISA plate. The plates are incubated for one hour at 37°C in a humid chamber and then washed (x 3) with PBS/T. Each well is immediately filled with 100 μl of peroxidase conjugated, affinity purified, monoclonal anti-human immunoglobulin G (H + L) conjugate labelled with horseradish peroxidase (HRP) or alkaline phosphatase (ALP) diluted at approximately 1:1,000 or 1:2,000 in PBS/T/M and incubated for one hour at 37°C. The optimum conjugate dilution is determined by checker-board titration. For canine VL rabbit anti-dog immunoglobulin G (H + L) conjugate is used. After incubation plates are washed again (x 3) with PBS/T and 100 μl of substrate solution added. The plates are left at room temperature for 15 minutes in the dark, after which the reaction is stopped by adding 50 μl of 2.5 M H₂SO₄. The results are read on an ELISA plate reader using a 492 nm filter within half an hour of stopping the reaction. All plates must include negative and positive control sera. Tests are normally performed in duplicate. Day to day variations in the test conditions can be adjusted with a positive reference sample as follows:

\[(\text{absorbance of the test sample/absorbance of the reference positive}) \times 1\]
APPENDIX 10

Leishmanin skin test (Montenegro)

Phosphate buffered saline (PBS, pH 7.2)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 gm</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Na₂HPO₄ 12H₂O</td>
<td>2.88 gm</td>
</tr>
<tr>
<td>KCL</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>up to 1 l</td>
</tr>
</tbody>
</table>

(This product can be made up at a concentration of 10 times the above recipe for better long-term storage).

Skin test diluent

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5 gm</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.75 gm</td>
</tr>
<tr>
<td>Phenol</td>
<td>4.0 gm</td>
</tr>
<tr>
<td>Distilled water (STERILE)</td>
<td>up to 1 l</td>
</tr>
</tbody>
</table>

Cultured *L. donovani*, *L. infantum* or *L. chagasi* promastigotes are washed (3 times) with sterile phosphate buffered saline (PBS) by centrifugation and re-suspension, preferably at 4°C. The final pellet is re-suspended in a skin test diluent at a concentration of between 5 x 10⁶ promastigotes/ml and 4 x 10⁸ promastigotes/ml. Concentrated stock solutions are diluted before use to 1 x 10⁶ promatigotes/ml. Both concentrated stocks and diluted antigens can be stored at 4°C for at least 12 months. Thiometersal may be used as an alternative to phenol for preventing contamination. In many countries leishmanin skin test antigens ready for use are available commercially.
0.1 ml of the antigen preparation is injected intra-dermally into the forearm of the patient (after the forearm has been cleaned with 70% alcohol). Control tests using solvents should be injected intra-dermally into the opposite forearm. The diameter of induration at the site of inoculation is measured at between 48 and 72 hours after inoculation using the ball-point pen method. The induration may be outlined with a ballpoint pen and the ink "lifted" by applying a piece of sticky tape; the tape with the ink mark provides a permanent record for each patient. (A piece of paper moistened with alcohol may be used instead of sticky tape.) A mean diameter of > 5 mm is considered to be a positive skin test reaction.

The leishmanin skin test is normally negative in patients with active VL but a positive reaction is associated with clinical cure or may be found in asymptomatic patients who have presumably been exposed to infection.

Comparison of previous studies in which skin testing was used is difficult because of the lack of uniformity in type and dose of antigen used. The use of well-standardized and evaluated antigens is important. The development of skin test antigens under the auspices of WHO is a significant progress in this respect.
APPENDIX 11

Drug supplies, costs and import restrictions

11.1 PENTAVALENT ANTIMONIALS

- Sodium stibogluconate (100 mg/ml)

  - Wellcome, Hale Court Greencourts Business Park, Styal Road, Manchester, M22 5LQ, United Kingdom.
  - Tel: +44.161.435.93.72. FAX: +44.161.435.93.63.

  * Average cost for VL treatment: **US$ 150**
  (20 mg Sb5+/kg/day for 30 days and average weight of 60 kgs)

- Meglumine antimoniate (85 mg/ml)

  - Rhône-Poulenc Rorer, 20 Avenue Raymon Aron, 92165 Antony Cedex, France.
  - Tel: +33.1.40.91.61.23 / +33.1.40.91.71.44.
  - Fax: +33.1.40.91.70.91.

  * Average cost for VL treatment: **US$ 120**

- Rhône-Poulenc Rorer, Avenida de Leganes, 62 Apartado 196, 28925 Alcorcon, Madrid, Spain.
  - Tel: +34.1.685.82.00.
  - Fax: +34.1.643.16.88.

  - Tel: +55.11.546.68.22
  - Fax: +55.11.523.69.91
- Sodium antimony gluconate (100 mg/ml)*

- *Albert David Ltd. 4/11 Asaf Ali Road,
  New Delhi, 110002, India.
  Tel: +91.11.32.77.667. FAX: +91.22.32.70.220
  or +91.22.32.82.675.

- *Albert David Ltd. 5/11 D. Gupta Lane,
  Calcutta 50, India.

  ° Average cost for VL treatment: **US$ 16**

- *Anoco Pharmaceuticals*, Private L.t.d. Brahmapuria,
  P.O.M.I.T., Muzaffarpur, Bihar state, 842003, India.

  ° Average cost for VL treatment: **US$ 16**

11.2 SECOND-LINE DRUGS

- **Amphotericin B (50 mg/unit)**

- *Bristol, Myers, Squibb, Pharmaceuticals Ltd.*
  Moreton, Wirral, Merseyside, L46-1QW
  United Kingdom.
  Tel: +44.151.604.23.24
  Fax: +44.151.604.01.89

- *Bristol, Myers, Squibb, Africa Division*
  Les Collines de l'Arche
  92057 Paris La Defense Cedex, France.
  Tel: +33.1.40.90.63.49
  Fax: +33.1.40.90.91.43
  +33.1.40.90.91.90

  ° Average cost for VL treatment: **US$ 60**
  (if total dose: 1g)

- **Aminosidine sulphate (500 mg salt/unit)**

- *Pharmacia and Upjohn*
  Via Robert Koch 12
  20152 Milan, Italy.
  Tel: +39.2.4838.1
  Fax: +39.2.4838.2734
° Average cost for VL treatment: **US$ 50**
(if 20 mg/kg/day for 21 days and average weight of 60 kgs)

- **Pentamidine isethionate (200 mg/unit)**

  - **Sedapharme**
    17, rue d'Orleans
    92200 Neuilly sur Seine, France.
    Tel: +33.1.47.45.05.06
    Fax: +33.1.47.45.39.23

° Average cost for VL treatment: **US$ 70**
(if 4 mg/kg on alternate day)

* Quality control currently under evaluation.*
APPENDIX 12

Insecticides and their use

Important characteristics of formulated residual insecticides are:

- High biological toxicity for the vector species. A WHO test kit with instructions for testing the susceptibility of sandflies to insecticides is available

- Repellent or irritating effect as low as possible

- Low acute and/or chronic toxicity to humans and domestic animals. When applied correctly, the risk of contaminating the outdoor environment is minimal

- Stability during storage, good mixing properties and non-corrosive for spraying material

- All the above properties at low cost

These criteria are important in choosing a suitable active ingredient and formulation. Several insecticides fulfil these criteria:

12.1 Chlorinated hydrocarbons

DDT is still considered a good choice for residual house spraying to control endophilic sandflies in a cost-effective way. The compound is stable, low cost, highly efficient, of long residual action and relatively safe for operators and inhabitants of sprayed houses. DDT, WP 75% is applied at 1 or 2 gm a.i./m². The susceptibility of sandflies to DDT is still very high. Nowadays the use of DDT has been banned in many countries for environmental reasons. DDT is manufactured in a limited number of countries. Apart from politico-ecological considerations, the status of DDT should be made clear vis-à-vis other insecticides taking into account that new compounds offer operational advantages and could be more cost-effective than DDT.

12.2 Organophosphorous insecticides

- Spraying activities should not exceed five hours per day and cholinesterase activity must be checked.

- Malathion, WP 50% is applied at 2 gm a.i./m². The slight smell emitted by this compound may reduce acceptability by the community.
3. Synthetic pyrethroids

Photo-stable pyrethroids show remarkably high toxicity for sandflies but low mammalian toxicity. They are used for residual house spraying, individual protection and space sprays.

- **Residual house spraying**

  Among others, the following pyrethroids are used:

  - deltamethrin: FW 5% for a t.d. of 25 mg a.i./m²,
  - permethrin: WP 25% for a t.d. of 125 mg a.i./m²,
  - cypermethrin: WP 10% for a t.d. of 30 mg a.i./m²,
  - cypermethrin: ME 10% for a t.d. of 30 mg a.i./m².

- **Impregnated bednets**

  Four compounds are currently used in demonstration projects:

  - deltamethrin: EC 2,5% for a t.d. of 25 mg a.i./m²,
  - permethrin: EC 25% for a t.d. of 500 mg a.i./m²,
  - lambda-cyhalothrin: EC 2,5% for a t.d. of 25 mg a.i./m²,
  - cypermethrin: EC 10% for a t.d. of 100 mg a.i./m².

The objective is to reduce the two main factors of vectorial capacity, that is, contact between humans and the vector and the lifespan of the vectors. Contrary to non-impregnated bednets where vectors are diverted to unprotected people, impregnated bednets will act as baiting traps. Pyrethroids induce an early knock-down effect on sandflies coming into contact with treated surfaces. The use of impregnated bednets will of course provide individual protection. A good coverage is required to obtain an impact on vectorial capacity, transmission and indirectly on the disease burden in the population.

Photo-stable pyrethroids are particularly appropriate for impregnation of bednets because of their long persistence and their relatively safety for humans. Permethrin, deltamethrin, lambdacyhalothrin and cypermethrin are currently under evaluation as potential alternatives for vector control in anthroponotic foci of leishmaniasis. Permethrin is more active on polyester and nylon than on cotton, while there is little difference between fabrics when deltamethrin is applied.
However, polyester or a mixture polyester/cotton will be preferred to cotton, because of their durability.

About half the dose of pyrethroids will be removed after the impregnated nets are washed in cold soapy water. Actually the washing of bednets causes a great loss of insecticide. If washed, nets have to be retreated.

- **Ultra Low Volume**

Spraying is performed with cold aerosol sprays (ultra low volume). Sandflies come into contact with the small droplets of insecticide suspended in the air when flying. The killing effect of such an application is fast but very short. Bad meteorological conditions could also compromise the final impact. This technique can be used on exophilic vectors during epidemic outbreaks.

The efficacy of chemical vector control in reducing the vectorial capacity depends more on the local vector ecology and behaviour than on the choice of one particular insecticide. Moreover, frequency of application by scheduling spray or reimpregnation rounds should be synchronized with seasonal peak periods of transmission.

* The above text is adapted from:
  CTD/MAL/SG/VC/BG/93.1 and
  CTD/MAL/SG/VC/WO/93.9
ABBREVIATIONS

a.i. active ingredient
ALAT alanine aminotransferase
ALP alkaline phosphatase
ASAT aspartate aminotransferase
CMV cytomegalovirus
DAT direct agglutination test
DCL diffuse cutaneous leishmaniasis
EC emulsifiable concentrate
ECG electrocardiograph
ELISA enzyme linked immunosorbent assay
ESR erythrocyte sedimentation rate
FCS foetal calf serum
Hb haemoglobin
HIV human immunodeficiency virus
IFAT indirect immunofluorescent antibody test
ME microencapsulated
PCV packed cell volume
PBS phosphate buffered saline
PKDL post kala azar dermal leishmaniasis
SRE systemic reticuloendothelial system
t.d. target dose
ULV ultra low volume spraying
VL visceral leishmaniasis
WBC white cell count
WP wettable powder
cc cubic centimetre
cm centimetre
dl tenth of a litre (100 ml)
g g force (for centrifugation)
gram
hr hours
kg kilogram
l litre
μl microlitre
M molar
mg milligram
ml millilitre
mm millimetre
mMol millimolar
min minutes
vol volume
w/v weight per volume
ILLUSTRATIONS

List of slides (In the order they are cited) and slide credit

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2. Prolonged irregular fever in human VL (Bryceson)
3. Nodular skin lesions in PKDL (El Hassan)
4. Skin colour changes in PKDL, Sudan (El Hassan)
5. Extensive facial lesions in late PKDL, Sudan (El Hassan)
6. Extensive facial lesions in late PKDL, China (Bryceson)
7. A sandfly on skin (Meddie)
8. Sandfly bites (Vexenat)
9. Individual protection through insecticide impregnated bednets (Desjeux)
10. An insecticide impregnated mosquito net may give protection against sandfly bites
11. Abundant sandflies (Lutzomyia longipalpis) may be found in chicken sheds (Vexenat)
12. Pig sties may also be infested with large numbers of Lutzomyia longipalpis (Vexenat)
13. Residual insecticide spraying on animal dwellings (Desjeux)
14. An apparently healthy dog with asymptomatic canine VL. Large number of amastigotes were found in the skin at the edge of the ears (Miles)
15. Canine VL: hair-loss around the eyes, muzzle and ears (Vexenat)
16. Skin lesions and severe emaciation in canine VL (Vexenat)
17. Skin ulcers at the border of the mouth in canine VL (Vexenat)
18. Elongated and deformed claws in canine VL (Desjeux)
19. Purulent discharged from the eyes in canine VL (Alvar)
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21. Blood spots on filter paper collected for serological tests (Desjeux)
22. NNN culture medium (Alvar)
23. Aspiration from the lymph node (Davidson)
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28. Leishmania promastigotes growing in culture medium (Alvar)
29. Inversion (A) of the albumin/globulin ratio in human VL, restored after treatment (B) (Bryceson; Meddia)
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