

**LEISHMANIOSIS****SUMMARY**

*Leishmaniosis is not a single entity but comprises a variety of syndromes due primarily to at least 16 species and subspecies of Leishmania. Dogs are commonly affected by L. infantum and L. chagasi (now regarded as synonyms), but canine infections with L. tropica, L. major and L. braziliensis have also been reported. In humans, the clinical spectrum ranges from asymptomatic infections to those with high mortality, with three distinct forms being classically described: visceral (VL), cutaneous (CL) and mucocutaneous (MCL). The vectors of these diseases are phlebotomine sandflies belonging to the genera Phlebotomus and Lutzomyia.*

**Identification of the agent:** *When clinical signs and characteristic lesions are present in affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates, of skin scrapings, and in tissue biopsies, gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting in-vitro or in-vivo isolation or by polymerase chain reaction (PCR). As there are very few morphological differences among various species, any isolated Leishmania organism must be identified by molecular, biochemical and/or immunological methods. Several centres throughout the world are presently using isoenzyme, DNA and antigen characterisation to identify the agent.*

**Serological tests:** *Serology is the preferred method for diagnosis of canine leishmaniosis and VL, even during the early stages of the disease. In subclinical forms, seropositive cases are confirmed by parasitological diagnosis or PCR. Serology is of less value for CL and MCL. Of the several serological techniques available, the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay are the most suitable. Serodiagnostic antigens need to be prepared in the laboratory, though some commercial products are under evaluation.*

**Delayed hypersensitivity test:** *The leishmanin skin test is useful for determining the distribution of human infections, distinguishing immune from nonimmune cases. The test is positive in CL, MCL and cured VL, but negative in active VL.*

**Requirements for vaccines and diagnostic biologicals:** *There is no effective vaccine available at present for use in dogs or humans. Leishmanin is no longer available commercially and needs to be standardised.*

**A. INTRODUCTION**

Leishmaniosis is caused by the vector-borne protozoan parasite, *Leishmania*. Various forms of clinical manifestations of human leishmaniosis have been described and divided into three entities: visceral leishmaniosis (VL, kala azar), cutaneous leishmaniosis (CL, oriental sore, uta, pian bois, chiclero's ulcer) and mucocutaneous leishmaniosis (MCL, espundia) (50). In the New World<sup>1</sup>, leishmanioses are caused by *L. braziliensis* complex (MCL and CL), *L. mexicana* complex (CL), *L. peruviana* (CL) and *L. infantum* (VL and CL); in the Old World, the aetiological agents are *L. donovani* (VL), *L. infantum* (VL and CL), *L. tropica* (CL), *L. major* (CL) and *L. aethiopica* (CL). *Leishmania infantum* and *L. chagasi* have been found to be identical by biochemical genotyping and should be regarded as synonyms (29). The diseases are mainly zoonoses with two exceptions, that of CL due to *L. tropica* in urban areas of Near and Middle East, and that of VL due to *L. donovani* the Indian sub-continent (northern India, Nepal and Bangladesh). Canine leishmaniasis (CanL) is a chronic viscerocutaneous disease caused by *L. infantum* (= *L. chagasi*), for which the dog acts as the source reservoir. In some instances, parasites belonging to *L. braziliensis* complex, *L. major* and *L. tropica* have been isolated from this host (31, 40). The

<sup>1</sup> In this chapter, the term 'New World' refers to the Americas, and the term 'Old World' refers to Europe, Africa and Asia.

vectors of leishmanioses are phlebotomine sandflies belonging to the genera *Lutzomyia* (New World) and *Phlebotomus* (Old World).

## B. DIAGNOSTIC TECHNIQUES

### 1. Identification of the agent

Clinical examination of suspected cases, parasitological diagnosis and immunodiagnosis are the routine methods available for the diagnosis of leishmaniosis. However, the demonstration of the parasite is the only way to confirm the disease conclusively. In VL and CanL, isolation and identification of the parasite from biopsies (lymph node, bone marrow, and spleen aspirate) coupled with molecular and immunodiagnostic tests are recommended. Parasitological diagnosis is necessary for confirmation of CL (through lesion scraping or needle aspiration from the edge of the lesions) as neither clinical examination nor serology is adequate. Smears of biopsy material are stained with Giemsa stain and examined microscopically at  $\times 600$ – $1000$  magnification. Material should also be cultured in appropriate media at  $22$ – $26^{\circ}\text{C}$ .

Morphological characteristics of amastigotes (in humans and mammalian hosts) and promastigotes (in phlebotomine sand flies and in cultures) are the following:

- *Amastigote*: small intracellular rounded or oval body,  $1.5$ – $3 \times 2.5$ – $6.5 \mu\text{m}$  in size, found in vacuoles within the cytoplasm of the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body;
- *Promastigote*: elongated extracellular organism, body size  $15$ – $20 \times 1.5$ – $3.5 \mu\text{m}$  with a single flagellum  $15$ – $28 \mu\text{m}$  long, arising close to the kinetoplast at the anterior. The nucleus is situated centrally.

The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the laboratory staff (45). *In-vitro* isolation offers certain advantages over the *in-vivo* methods: cultures become positive more rapidly (5–30 days compared with months for lesions to appear on an animal) and the materials are less expensive. However, for *in-vitro* isolation, the techniques used should be carried out under strictly sterile conditions, which is rarely feasible in the field. Unfortunately, there is still no 'universal' culture medium in which all the different leishmanias will grow easily, and it is almost impossible to predict which medium will be best suited to the growth of a particular isolate of *Leishmania*. Individual laboratories have to find the most suitable medium among biphasic blood agar media and tissue culture media supplemented with fetal calf serum (14). When attempting primary isolation of unknown organisms, a blood agar-based medium should be used – preferably NNN medium (Novy, McNeil and Nicolle), otherwise brain–heart infusion (BHI) agar medium or EMTM (Evan's modified Tobie's medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.a (see ref. 14 for media composition). The organisms from patients with VL and MCL can be very difficult to cultivate. The parasites sometimes die when subcultured, even when the initial isolation is successful. This seems especially common when the initial isolation has been into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media, such as NNN, or one of the semisolid media such as 'sloppy Evans' or semisolid Locke blood agar.

Hamster (*Mesocricetus auratus*) is the most commonly used animal for *in-vivo* isolation. Tissue suspensions or aspirates are inoculated intradermally into the nose and/or feet in the case of detection of dermatropic parasites. When the material is suspected to be infected with parasites causing VL, the inoculation should preferably be made by the intraperitoneal route. The resulting infection becomes apparent, weeks or months later, by the development of a nodule or ulcer at the site of inoculation, and in case of viscerotropic parasites, the infection becomes apparent, some months later, by massive infection of internal organs. The examination of Giemsa-stained smears of hamster tissue suspension/aspirate will show amastigotes. BALB/c mice are commonly used for the diagnosis of *L. major*.

Several techniques are now being used in many centres to identify the different *Leishmania* species, subspecies or strains.

- a) Isoenzyme characterisation, also known as MLEE (multi-locus enzyme electrophoresis), is the reference method for species identification (23, 39, 45, 50), although this technique requires cultivation of a large number of parasites ( $5 \times 10^9$ – $1 \times 10^{10}$ ). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evan's blood lysate broth] medium, Schneider's *Drosophila* medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in the matrix towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain,

many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

- b) The monoclonal antibody (MAb) technique is applied to the analysis and classification of *Leishmania* species and subspecies (20). For the production of the antibodies, BALB/c mice are immunised with membrane preparations from either promastigotes or amastigotes. Antibody-secreting hybridoma cultures are then selected and cloned by limiting dilutions. Specificity to *Leishmania* strains is assessed through immunofluorescence or immunoradiometric assays. This analysis should be quantitative, as the amount of the same surface antigen may vary among *Leishmania* species. Monoclonal antibodies have also been used in immunohistochemical techniques applied to tissue biopsies.
- c) DNA hybridisation probes are a very specific tool the principle of which is to allow labelled, single-stranded nuclear or kinetoplast DNA sequences from well characterised standard strains to find and hybridise with homologous DNA sequences from or within unknown *Leishmania* isolates (19, 44). Only complementary DNA sequences will form double-stranded DNA, which can be detected by autoradiography if the probe is radiolabelled, or by immunoenzymatic reaction. These techniques are sensitive enough to identify  $10^2$ – $10^3$  organisms spotted on to nylon filters. Much fewer parasites (<10) are required for identification through the *in situ* hybridisation technique.
- d) Polymerase chain reaction (PCR)-based methods are available for diagnosis and/or identification of *Leishmania* from different types of human and canine samples. Essentially, techniques developed either to detect organisms from fresh or frozen, formalin-fixed and paraffin-embedded biopsies, or to identify established isolates of *Leishmania* include: (a) digestion of material with proteinase K and DNA extraction; (b) standard PCR amplification using oligonucleotide sequences (primers) selected from the small-subunit rRNA gene (28), kinetoplast DNA minicircles (25) or other highly repetitive genomic DNA sequences (9, 36); (c) analysis of amplification products by 1–2% agarose gel. To increase sensitivity, a nested or semi-nested PCR using internal primers from the above sequences can be performed. In human VL, PCR has a sensitivity comparable with that of culture-based methods, but gives results much faster. In CanL, the diagnostic efficacy of PCR as compared with serology depends on the natural course of the disease, the sensitivity being highest shortly after infection (37). In American CL and MCL, PCR appears to be consistently more sensitive than any previously recommended method of diagnosis (13). Different techniques have been described that improve both sensitivity and specificity of the method, such as the PCR-RFLP (restriction fragment length polymorphism) analysis in which the PCR products are digested by appropriate restriction enzymes and the resulting restriction fragment pattern is analysed for species or strain identification (30, 46). Real-time PCR methods, which allow the continuous monitoring of the accumulation of PCR products during amplification, have been described and are available commercially. They can be more sensitive than conventional PCR, and are mainly addressed to study the kinetics of infection and monitoring therapeutic response (3, 7). In addition, real-time PCR has been reported to be useful for evaluating infections in less invasive samples such as blood (15).

## 2. Serological tests

Several serological tests are used for detecting anti-leishmanial antibodies. Sensitivity values reported below for each test, however, apply only to individuals who are not immunocompromised. A high percentage of patients with VL co-infected with human immunodeficiency virus (HIV) have been reported to be seronegative for anti-leishmanial antibodies (18).

### a) Indirect fluorescent antibody test

The indirect fluorescent antibody (IFA) test is widely used because it is easy to perform. The test is genus specific, although significant cross-reactions have been reported in individuals infected with *Trypanosoma cruzi*. For these subjects, serological tests based on specific recombinant *Leishmania* antigens would be more appropriate (see Section B.2.b and d below). In Chagas' disease-free areas, the IFA test for the diagnosis of clinical VL or CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA. Although amastigotes from frozen sections or smears of infected organs can be used as antigen, cultured promastigotes represent the commonest antigen source.

#### o Antigen preparation

- i) Harvest 3–4 ml of the liquid media of a 3-day-old culture showing flourishing promastigote growth (see Section B.1 for culture media).

- ii) Wash the organisms three times with phosphate buffered saline (PBS), pH 7.2–7.4, by centrifugation at 350 **g** for 15 minutes at room temperature.
- iii) Resuspend the final cell pellet in PBS and adjust the promastigote concentration to approximately  $4 \times 10^6$ /ml with the aid of a haemocytometer.
- iv) Distribute 30  $\mu$ l of the promastigote suspension on to each circle of a multispot slide and allow to dry at room temperature.
- v) Fix the promastigotes in cold acetone for 10 minutes, then put the slides into a plastic box and keep in a deep freezer ( $-35^\circ\text{C}$ ) for no longer than 2–3 months.
- o **Test procedure**
  - i) Wash the frozen antigen-coated slides in PBS and allow to dry at room temperature.
  - ii) Inactivate the sera for 30 minutes in a water bath at  $56^\circ\text{C}$ .
  - iii) Make doubling dilutions of test sera from 1/80 to 1/10,240 for human VL, and from 1/40 to 1/5120 for CanL. Positive and negative control sera, at dilutions of 1/80 and 1/160 for human VL, and of 1/40 and 1/80 for CanL, are also included in the test. No standard sera are available, but internal standards should be prepared and titrated.
  - iv) Distribute 30  $\mu$ l of diluted serum samples on to each slide circle and incubate for 30 minutes at  $37^\circ\text{C}$ .
  - v) Remove the serum samples by vigorous washing in PBS, followed by immersion of the slides in PBS for 10 minutes. Allow the slides to dry.
  - vi) Distribute 30  $\mu$ l of diluted fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin on to each slide circle and incubate for 30 minutes at  $37^\circ\text{C}$ . FITC-conjugated anti-human and anti-dog immunoglobulins are commercially available. Follow the instructions for the appropriate dilution.
  - vii) Repeat step v and mount with a cover-slip in a few drops of PBS/glycerol (50% [v/v] of each).
  - viii) Read the slides under a fluorescent microscope. The highest dilution showing fluorescent promastigotes is taken to be the antibody titre. In human VL, the threshold titre usually ranges from 1/80 to 1/160, while in CanL it ranges from 1/40 to 1/160. As IFA test performance may vary in different laboratories, it is better for each laboratory to define its own threshold titre using defined positive and negative reference sera.

**b) Enzyme-linked immunosorbent assay**

The ELISA can be carried out on serum or on a measured volume of blood. The blood is collected by needle-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined to give an acceptable sensitivity and specificity. This test can be used for seroepidemiological surveys under field conditions.

In the classical method, the antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS, pH 7.2, at 1000 **g** for 15 minutes. The packed promastigotes are resuspended in twice their volume of distilled water, and then sonicated at medium amplitude in an ice bath. The suspension is left at  $4^\circ\text{C}$  overnight to allow the proteins to come into solution. After a final centrifugation at 4000 **g** for 10 minutes to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at  $-20^\circ\text{C}$  until required. For use in the test, it is reconstituted with PBS to the predetermined optimal protein concentration (around 20  $\mu\text{g}/\text{ml}$ ) as measured by Lowry's method. The ELISA is useful for the diagnosis of Old and New World leishmanioses. There is little or no cross-reaction with other diseases and, according to the *Leishmania* strain used, sensitivity can range from 86% to 99%.

A version of the ELISA called the Falcon assay screening test and enzyme-linked immunosorbent assay (FAST-ELISA) and which uses antigen-coated beads, is considered to be a sensitive, specific and field-adaptable test for visceral CanL with comparable sensitivity and specificity to the IFA test and ELISA. Whole blood or plasma can be evaluated quickly without the use of a microscope or spectrophotometer (1).

A detergent-soluble promastigote antigen has been used in ELISA instead of the crude lysate, for the diagnosis of CanL. The detergent was Triton X-100 and the proteic extract was protected with protease inhibitors. Using this method, ELISA sensitivity increased to 99.5%, while its specificity was comparable with that of the IFA test (97%) (26).

The ELISA methods described above are all based on crude antigenic preparations. More recently, a recombinant antigen from a cloned protein of *L. infantum*, called rK39, has been reported to be highly reactive to sera from human and canine visceral leishmaniosis cases when run in an ELISA format. Using 25–50 ng of the antigen, 99% specificity and sensitivity was consistently found for immunocompetent human

patients with clinical VL and for dogs with parasitologically proven disease (2, 41). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (24). The K39 antigen, which shows remarkable stability and reproducibility, is now produced commercially.

#### c) Direct agglutination test

The direct agglutination test (DAT) has been described for the diagnosis of VL and CanL. After test improvement, DAT has been validated as a specific and sensitive assay for field investigations (4, 10, 35). The antigen consists of promastigotes harvested from cultures, washed in PBS, pH 7.2, treated with 0.4% trypsin (for 45 minutes at 37°C and then washed again), and stained with 0.02% Coomassie brilliant blue. Twofold serial dilutions of serum in PBS are made in V-bottomed microtitre-plate wells; 50 µl of antigen preparation is added to each well, and the plate is then carefully shaken by hand and left for 18 hours at room temperature. The test is read visually against a white background. Positive reactions are indicated by a clear sharp-edged blue spot.

A modified DAT for detection of specific anti-leishmanial antibodies in canine reservoir hosts is considered to be highly suitable for wide-scale epidemiological and ecological field work and diagnosis of CanL, having 100% sensitivity and 98.9% specificity (21, 22). The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C.

#### d) Rapid immunochromatographic assay (dipstick or strip-test)

A rapid immunochromatographic assay using rK39 as antigen (K39 dipstick or strip-test, commercially available) has been evaluated in different endemic settings of VL. The nitrocellulose membrane of the test kit holds an absorbent pad at one end, a band of immobilised anti-protein A antibody (used to detect IgG) at the other (control region), and a band of rK39 antigen in the middle (test region). A protein-A-colloidal gold conjugate is used as the immunochromatographic detection reagent. One small drop (20 µl) of the serum to be examined is placed on the absorbent pad before two large drops (100 µl) of test buffer are added to the pad, and the mixture is allowed to migrate up the strip by capillary action. After 2–10 minutes, the result is positive if two distinct red lines appear (one in the test region and another in the control region), it is negative when no red line appears in the test region, and it is invalid if the control line fails to appear.

In clinical cases of human VL, the K39 dipstick showed 100% sensitivity and 93% specificity in India (43), 90% sensitivity and 100% specificity in Brazil (11), and 100% sensitivity and specificity in the Mediterranean basin (8). In parasitologically proven CanL, in both asymptomatic and symptomatic cases, the sensitivity of the K39 dipstick was 97% and the specificity 100% (34).

### 3. Delayed hypersensitivity test

Delayed hypersensitivity is an important feature of all forms of human leishmaniosis and can be measured by the leishmanin test, also known as the Montenegro reaction (27). The leishmanin skin test has no value for the diagnosis of CanL. Leishmanin is a killed suspension of whole ( $0.5\text{--}1 \times 10^7/\text{ml}$ ) or disrupted (250 µg protein/ml) promastigotes in pyrogen-free saline containing phenol. A delayed reaction develops and is read at 48–72 hours.

The false-positive reaction rate in otherwise healthy people is approximately 1%, but this can be higher in areas where there is a background of leishmaniosis, as many of the healthy population may show quite high rates of leishmanin sensitivity. Although there is complete cross-reactivity among all strains of *Leishmania*, although heterologous antigens often give smaller reactions, which may be caused by difficulty in standardisation. The leishmanin test is used in the clinical diagnosis of CL and MCL. In VL it will only measure past infections because during active disease, a complete anergy is found. Leishmanins are not available commercially.

## C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

### 1. Vaccine

There is no effective vaccine available for prophylactic immunisation against leishmaniosis. Until now, the only dependable vaccination against *Leishmania* has been limited to the protection of humans from both *L. tropica* and *L. major* by prior syringe-induced infection with *L. major* organisms. The promastigotes are injected into the arm or other parts of the body. The living promastigotes used must either be freshly extracted from cultures or may be preserved in liquid nitrogen. The infection is allowed to run a natural course and after recovery, the individual is firmly immune to subsequent infection with both *Leishmania* species. This type of immunisation has been practised on a limited scale in hyperendemic areas of CL (due to *L. major*) in Israel, Iran and the former Union of Soviet Socialist Republics (42). *Leishmania major* causes cross-protection against *L. tropica*, but the reverse is probably not true. However, this species cannot be considered to be totally safe and this type of immunisation should be used only for humans moving into high-risk areas. Moreover, it is not beneficial in highly endemic areas as individuals contract infection long before this type of preparation confers protection (i.e. approximately

3 months after vaccination). Standardisation and quality control of such vaccines, presently not available, are needed.

At present, a number of promising anti-leishmanial vaccines are under development (12, 16). Among the first-generation vaccines, the glycoprotein-enriched fraction of *L. donovani* known as 'fucose-mannose ligand' (FML), developed in Brazil, represents the first licensed veterinary vaccine against CanL. Field studies showed about 80% clinical protection conferred by the antigen administered with QuilA saponin as adjuvant (5), and also good immunotherapeutic efficacy when used in sick dogs (6). Killed *Leishmania* organisms mixed with a low concentration of BCG as adjuvant have undergone phase I–II and phase III trials for immunisation against CL *Leishmania* agents in humans and against VL in humans and in dogs, with limited success (32, 33).

Second-generation vaccines, most of which are at predevelopment stage, consist of genetically reconstructed *Leishmania* parasites incapable of producing disease, recombinant molecules or their corresponding DNAs, or recombinant organisms carrying leishmanial genes and expressing parasite antigens. A chimeric antigen generated from three recombinant *Leishmania* antigens screened for their ability to elicit cellular immune responses (known as Leish-111f), entered Phase I clinical testing in healthy volunteers in January 2003 (38). The same polyprotein antigen, administered with monophosphoryl lipid A – stable emulsion (MPL-SE) or Adjuprime as adjuvants, failed to protect dogs from *L. infantum* infection in a phase III trial (17).

## 2. Immunodiagnostic antigens

Neither the leishmanin used for skin tests nor the antigens commonly employed in serodiagnosis in leishmaniosis are internationally standardised (the recombinant K39 antigen, which is virtually standardised, is patent-protected and is not widely available). The leishmanin test is group-specific, not species-specific, and the leishmanin prepared from one clinical type of leishmaniosis will cause the development of delayed hypersensitivity to the same or other clinical types. Similarly, serological cross-reactions are common among leishmanial species.

### a) Leishmanin

The leishmanin test is described in Section B.3. Sterility, safety and potency tests are required for leishmanin preparations.

### b) Antigens for serological tests

Commercial antigens for the IFA tests and ELISAs have been produced are still under evaluation. The main reason for unsatisfactory results with these antigens is the poor stability of leishmanial antigens. They can be obtained in the laboratory by growing a *Leishmania* strain in a suitable culture medium. For the IFA test and the DAT, crude particulate antigens, i.e. intact promastigotes, are required, whereas for ELISAs a soluble form of the antigen is needed.

## 3. Seed management

### a) Characteristics of the seed

Strains of *Leishmania* species used to prepare biological products should be identified at species and subspecies level by appropriate identification tests given in Section B.1. Once the organisms have been isolated and established in the laboratory, they must be assigned an International Code (45, 50). This Code should consist of four elements separated by oblique strokes: (a) the type of host from which the strain was isolated (M for Mammalia and I for Insecta followed by three letters indicating the generic name of the host); (b) the country where isolation was made, indicated by a two-letter code; (c) the year of isolation indicated by the last two digits, and (d) the original laboratory code given to the isolate (for example, MHOM/IN/80/DD8). The parasites must be free from contaminating organisms and should be capable of yielding a product that conforms to the norms. Standard strains are available on request from the World Health Organization (WHO) Collaborating Centres in Madrid (Spain), Montpellier (France) and Jerusalem (Israel). A list of Identification Centres has been published by WHO (50).

### b) Method of culture

The strain of the parasite used for preparing leishmanin should be capable of producing a product that conforms to national/international norms. It should be free from ingredients causing toxic or allergic reactions. There is no single specific antigen standardised for use in serodiagnostic tests, but when these antigens are prepared in the laboratory, they must be standardised for their sensitivity depending on the requirement. For the preparation of leishmanin as well as serodiagnostic antigens, the organisms should be grown in a suitable culture medium (such as those recommended in Section B.1 for *Leishmania* isolation and bulk cultivation). Normally, good growth of parasites is obtained 7 days after inoculation, and care must be taken that leishmanial stocks are not lost by overgrowth of the flagellates, which may occur after approximately 10 days.

**c) Cryopreservation**

Promastigote cultures and tissue infected with amastigotes may easily be conserved in the living state at low temperatures. Both forms can be cryopreserved for years at low temperatures in mechanical freezers ( $-70^{\circ}\text{C}$ ), in solid carbon dioxide containers ( $-76^{\circ}\text{C}$ ), or in liquid nitrogen containers ( $-196^{\circ}\text{C}$ ) (45). A sterile cryoprotectant is required – glycerol, to give a final concentration of 7.5–10%, or dimethyl sulphoxide (DMSO), to a final concentration of 5–7.5%. The cryoprotected samples are transferred to the sterile containers in which they are to be frozen. These may be 2 ml plastic freezing tubes with airtight screw-caps, hard glass, heat-sealed ampoules, or glass/plastic capillaries. A slow cooling rate (approximately  $1^{\circ}\text{C}/\text{minute}$ ) is essential for the cryopreservation of *Leishmania*. This can be obtained by cooling samples to  $4^{\circ}\text{C}$  and keeping them at this temperature for a minimum of 1 hour; they are then transferred to a  $-20^{\circ}\text{C}$  freezer and left for 24 hours, then removed to a  $-70^{\circ}\text{C}$  freezer for at least 24 hours. They can be permanently stored at this temperature, or else transferred into liquid nitrogen or solid carbon dioxide. If possible, a programmable freezing unit should be used. When the cryopreserved material is required, the sample is taken out and thawed rapidly in a water bath at  $37^{\circ}\text{C}$ .

**d) Validation**

Cultures for leishmanin or serodiagnostic antigens should be checked for sterility before use. Leishmanin is stored at  $4^{\circ}\text{C}$  and serodiagnostic antigens at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  until required. The latter should be reconstituted with PBS, pH 7.2, before use. Viable *Leishmania* cultures can be kept at  $-70^{\circ}\text{C}$  for 3–4 years or at  $-196^{\circ}\text{C}$  indefinitely. Because of nonavailability of suitable vaccine, it has not been possible to validate the currently developed immunising agents. Live or attenuated promastigotes of *L. major* used in some areas are far from being satisfactory. Leishmanin should be tested for allergenicity in guinea-pigs before use. Serodiagnostic antigens should be tested for their efficacy and sensitivity by proper standardisation for a particular test. If a batch of antigen has not been used for a long time, it should be rechecked before being used in the test.

**4. Method of manufacture**

As standardised immunodiagnostic antigens are not available commercially, they need to be prepared in the laboratory. Workers in the laboratory can be at risk of laboratory acquired infection, especially by injection. Appropriate biosafety precautions are therefore essential to minimise the risks (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

**a) Leishmanin**

*Leishmania* species are grown, preferably in blood-free liquid media such as Schneider's *Drosophila* medium and RPMI (Rosewell Park Memorial Institute) medium, in order to avoid blood–antigen contamination. The promastigotes are harvested during the log phase, washed four times in pyrogen-free saline at 1000 **g** for 15 minutes, and resuspended in pyrogen-free saline containing 0.5% phenol (w/v) to obtain a final concentration of  $0.5\text{--}1 \times 10^7/\text{ml}$ . Leishmanin can also be made with disrupted promastigotes obtained as above and sonicated. The filtrate is adjusted to a final protein concentration of 250  $\mu\text{g}/\text{ml}$  with pyrogen-free saline containing Tween 80 (0.0005% [v/v]) and phenol (0.28% [w/v]).

**b) Antigens for serological tests**

Methods of antigen preparation for various tests are given in Section B.2.

**5. In-process control**

One or more batches of leishmanin should be tested in guinea-pigs by allergic test. Sensitivity and specificity of the leishmanin should preferably be determined by performing the test in appropriate animal models (different inbred mice according to the *Leishmania* species), or in patients who have recovered from leishmanial infections, and in an unexposed control population.

**6. Batch control**

The WHO has suggested guidelines for the production of leishmanin (48, 49). It is recommended that the source material be controlled by using isoenzyme analysis to type the *Leishmania* strains used in preparing leishmanin.

**a) Sterility**

Each filling lot should be tested for bacterial and mycotic sterility according to WHO (47). Absence of live leishmaniae is checked by inoculating one sample of each lot in an appropriate blood–agar medium, which is then incubated at  $23^{\circ}\text{C}$  for at least 15 days. One sample is injected intradermally (for dermatropic leishmaniae) or intraperitoneally (for viscerotropic leishmaniae) in mice or hamsters. These animals are observed during a period of 30–90 days.

**b) Safety**

Samples from each filling lot should be tested for abnormal toxicity by appropriate tests in guinea-pigs and mice. For each lot, five mice weighing 17–22 g and two guinea-pigs weighing 250–350 g are injected subcutaneously and intraperitoneally with one human dose of the product. The animals are then observed for at least 7 days for death or signs of disease.

**c) Potency**

The leishmanin is tested on animal models (according to the *Leishmania* species involved) that have been previously infected by the same strain used for leishmanin production. Lots of at least five infected animals and control animals are injected intradermally into one of the posterior footpads with 50 µl of leishmanin. After 2–3 days, all the infected animals should show a significant enlargement of the footpad compared with control animals.

**7. Tests on the final product****a) Safety**

See Section C.6.b.

**b) Potency**

See Section C.6.c.

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