Diagnostic criteria and principles of management of leishmaniasis

(GB 15986-1995)

These criteria are developed according to the *Law on the Prevention and Treatment of Infectious Diseases of the People's Republic of China* and *Measures for the Implementation of the Law on the Prevention and Treatment of Infectious Diseases of the People's Republic of China.*

1. Contents and scope of application

This document specifies the diagnostic criteria, treatment and prevention principles of leishmaniasis.

It is intended to be used by the technical organizations in the epidemic areas in leishmaniasis prevention and control as well as by healthcare facilities at all levels and of all types in diagnosing and managing leishmaniasis cases.

2. Diagnostic principles

Diagnosis is made based upon epidemiological history, clinical manifestations, parasitological and serological findings.

3. Diagnostic criteria

3.1 Leishmaniasis

Leishmaniasis is transmitted through sandfly’s bite. It mainly affects internal organs, with some cases mainly manifested with skin lesions or simple lymph node enlargement, which is called skin or lymph node type leishmaniasis. Dogs can be infected too (canine visceral leishmaniasis). Dogs are the main animal hosts in hilly leishmaniasis epidemic areas and they are mostly the local source of human infections.

3.1.1 A resident in leishmaniasis epidemic areas; or one ever living in such epidemic areas during May and September when sandflies are active.

3.1.2 Irregular fever for a long time; progressive enlargement of spleen; mild or intermediate enlargement of liver; decreased white blood cell count; anemia; thrombocytopenia; or hemorrhinia and gingival haemorrhage.

3.1.3 Leishmania amastigotes detected on smear of puncture biopsy from bone marrow, spleen or lymph nodes; or Leishmania promastigote detected on NNN culture from the above puncture biopsy (see Annex A for details).

3.1.4 Antibody positive found on IFA, ELISA, PVC film rapid ELISA and IHA; or circulating antigen positive on monoclonal antibody spot-ELISA (direct McAb spot-ELISA), monoclonal antibody-antigen spot test (McAb-AST) or monoclonal

antibody-EITB test (see Annex B for details).
Suspect case: compatible with both 3.1.1 and 3.1.2.
Clinically diagnosed case: suspect case plus compatible with 3.1.3.
Confirmed case: suspect case plus compatible with 3.1.4.

3.2 Skin type leishmaniasis
3.2.1 Most cases have a history of suffering from leishmaniasis several years ago or more than a decade ago, but it can also occur during the course of leishmaniasis. A few cases have no history of leishmaniasis and are primary cases.

3.2.2 Nodules, erythema and papules, sometimes discoloration of skin are seen on the face, limbs or trunk; white blood cell count may be up to 10000/mm^3 with eosinophil count often over 5%.

3.2.3 Leishmania is detected on smear from tissue fluid drawn from nodules or papule or on smear from skin scrapings.

3.2.4 Circulating antigen positive on McAb dot-ELISA.
Suspect case: compatible with 3.2.1 and 3.2.2.
Confirmed case: suspect case plus compatible with 3.2.3.
Clinically diagnosed case: suspect case plus compatible with 3.2.4.

3.3 Lymph node type leishmaniasis
3.3.1 Most cases are adult living in or traveling form non-epidemic areas to leishmaniasis epidemic areas (deserts or hills) where most cases are infants during the high season of sandflies.

3.3.2 Enlargement of cervical lymph nodes, retroauricular lymph nodes, axillary lymph nodes, inguinal lymph nodes or supratrochlear lymph nodes that are peanut-sized, shallow and movable. No enlargement of liver or spleen but with eosinophilia.

3.3.3 Tissue liquid from enlarged lymph nodes for detecting Leishmania on smear examination; or detecting Leishmania on lymph node slides.
Suspect case: compatible with 3.3.1 and 3.3.2.
Confirmed case: suspect case plus compatible with 3.3.3.

4 principles of management

4.1 Treatment (see Annex C for details)

4.1.1 leishmaniasis

4.1.1.1 Initial treatment cases:

Six-day Solustilbosan therapy: total dosage for adults: 120-150 mg Sb/kg; total dosage for children: 200-240 mg Sb/kg; 1/6 of total dosage per day, im or iv, for 6 days.
Three-week Solustilbosan therapy: total dosage for adults: 133 mgSb/kg; total dosage for children: 200 mg Sb/kg; 1/6 of total dosage each time, im or iv, twice a week, for 3 weeks. This therapy applies for severe patients or week patients.

4.1.1.2 Patients uncured or relapsing after a full course of treatment: increase the dosage of Solustilbosan of 6-day regimen by 1/3 to apply 8-day regimen.

4.1.1.3 For cases resistant to Solustilbosan, any of the following regimens can be used:
Pentamidine, 4mg/kg, q.d. or qod, im, total dosage: 60mg/kg;
Hydroxystilbamidine: 2-3mg/kg each time, im or iv, total dosage: 85mg/kg.

4.1.2 Skin type leishmaniasis
Solustilbosan six-day or eight-day therapies for 2-3 consecutive courses. Pentamidine is effective and can be given 4mg/kg, im, with total dosage of 60-80mg/kg to cure the case. If skin lesions still stay, one more course of therapy can be given.

4.1.3 Lymph node type leishmaniasis
Solustilbosan therapy: dosage and treatment course remain the same as in 4.1.1.1.

4.2 Prevention

4.2.1 Elimination of infected dogs: in hilly epidemic areas, use parasitological test and serological tests to find dogs with visceral leishmaniasis for culling. Public education to raise awareness of keeping fewer or no domestic dogs is needed in areas with large number of dogs.

4.2.2 Sandfly control: In leishmaniasis epidemic plain areas with high density of sandflies, in the beginning of the high season of sandflies, pesticides should be sprayed on human and animal houses. In desert or hilly areas during the sandfly high season, when a case is detected, spray pesticides in the affected house and human houses and animal houses within 15 meters around the affected house to eliminate the sandflies.

4.2.3 Sandfly preventive measures
Use mosquito nets or burn mosquito-repellent incense or dried mugwort to expel sandflies. Do not sleep in open places and window/door screens are recommended.
In hilly epidemic areas of leishmaniasis, pesticides (eg. deltamethrin) can be sprayed on dogs' body to kill or expel sandflies in the epidemic seasons. Personnel on night duty in wildness should use expellant on naked areas of body.

Annex A Pathogenic diagnosis (Supplement)

A1 Pathogenic examination

A1.1 Iliac puncture biopsy

A1.1.1 Patient lying on his/her side to expose ilium; locate iliac spine with fingers and sterilize the concerned skin with iodine and alcohol. Operate the procedure under local anesthesia.

A1.1.2 The size of the puncture needle is subject to the age of the patient: No. 20 puncture needles for infantile and young children; No. 18 lumber puncture needles for children and young adults; No. 17 puncture needles for adults. All should be sterilized with pressure steam.

A1.1.3 Puncture at 1cm post the anterior superior iliac spine. Insert the needle then further insert the needle at 70-80 degrees against the horizontal line through the subcutaneous tissue and periosteum. When the needle tip is felt to touch bone surface, rotate the needle into the bone.

A1.1.4 Depth of the puncture may vary between 0.5cm and1.0cm subject to the age and size of the patients. When the needle is let free from the hand and remains standing, it indicates that it is in the bone. Remove the needle axis and apply 2ml or 5ml syringe to get bone marrow. Then immediately pull out the puncture needle. Prepare smear of bone marrow for examination.

A1.1.5 Leave the bone marrow smear dry naturally and mark with numbers. Fix the smear with methanol before staining. Dilute Giemsa staining solution with water to 3% solution and stain for 30 min; or add 3 drops of Giemsa staining solution into 2ml water and drop the solution on the smear and stay for 20 min. Rinse with running water; dry up for microscopic exam (oil len).

A1.2 Lymph node puncture

A1.2.1 Groin is the most common site but enlarged lymph nodes on other sites can also be used for puncture.

A1.2.2 Enlarged lymph node is sterilized; pinch and fix a lymph node with clean thumb and index finger and raise it with caution of not contaminating the puncture site. Hold the high-pressure sterilized needle in right hand; puncture through skin until into the lymph node. Pull out the needle after a few seconds. Aspiration is not needed.

A1.2.3 Inject the lymph in the needle onto the slide to prepare carefully the smear.

A1.2.4 Microscopic examination on smear: see A1.1.5.

A1.3 Skin scraping smear

After sterilization of the skin, pinch the skin nodule with clean left thumb and index finger and cut gently the skin; scrape some skin tissues from the cut to make stained smear for microscopic examination.

A1.4 Preparation of NNN culture base: agar 14.0g, NaCl 6.0g and distilled water 900mL in a flask; heat to melt and divide into different tubes with 3mL in each tube.

After steam sterilization and cooling down, add 1/3 of defibrinated rabbit blood in each tube 1/3; mix and let cool. Then add 0.5mL of Locke's solution in each tube and keep at 4℃for future use.

A1.5 Leishmania culture: Under strict sterile condition, inject the collected bone marrow or lymph into the culture base, or put a small piece of skin tissue cut from the leishmaniasis case into the culture base. Leave the culture in the incubator at 22-24℃ for 15 days. Use a platinum loop to take some culture liquid for microscopic examination. Diagnosis is confirmed if Leishmania promastigote is seen.

Annex B Serological diagnosis (Supplement)

B1 Antibody test

B1.1 IFA

Serum is generally used for testing but for infantile cases, dried blood spot method can be used.

B1.1.1 Antigen slides: collect Leishmania promastigote from the NNN culture base after 10 days; centrifuge at 3000r/min for 15min; discard the upper clear liquid; add saline into the precipitate and mix well; centrifuge and rinse for 3 times; use 0.01mol/l pH7.2 PBS containing 0.2% formalin to fix and then leave in refrigerator for 1 hour; centrifuge and discard the upper clear liquid; rinse the precipitate with PBS once again to dilute to 50-100 promastigotes each field; drop on a slide and blow dry. Keep in -20℃freezer.

B1.1.2 Preparation of dried blood spot: draw a circle of 1.2cm diameter on a filter paper and drop 2 drops (about 20 mm^3) of patient's earlobe blood; let dry and put in a plastic bag with desiccant and keep in refrigerator for examination.

B1.1.3 Cut off the dried blood spot from the filter paper and soak it in 2ml of 0.01mol/l pH7.2 PBS solution (equivalent to 1:20 serum) (if the sample is serum, then dilute by 1:20) and put in refrigerator overnight. Continue to do double-dilution until 1:320 or 1:640; drop serum or dried blood spot soak solution of different dilution onto the slides; put in moisture container and put in 37℃incubator for 30 min; wash away slowly the serum or dried blood spot soak solution with pH 7.2-8.0 PBS; soak in PBS for 10 min; wash with distilled water once more and blow dry.

B1.1.4 Add 1∶10 fluorescent labeled sheep anti-human IgG; put in moisture container and put in 37℃incubator for 30 min; wash as before and blow dry.

B1.1.5 Add a drop of distilled water on the slide and cover with a slide; observe under fluorescence microscope or 6×40 times optical microscope with high pressure mercury fluorescent light. In positive cases, cytoplasm and flagella of the parasite have yellow-green fluorescence with clear margin while nucleus and kinetoplast generally do not have fluorescent.

B1.1.6 At each test, dried blood spot (or serum) of normal people and PBS are used as control to patient's dried blood spot (or serum). Since normal blood may occasionally be positive (+) at 1:20 dilution, "++" is used as the criteria for positivity and 1:20 as the minimal positive dilution.

B1.2 PVC film rapid ELISA

B1.2.1 Antigen (promastigote soluble antigen): collect Leishmania promastigote and centrifuge in saline and wash for 3 times; add 10 times of 0.01% thiomersal saline and give ultrasonic treatment in ice bath twice, 10 min each time, freeze thawing for 5 times; centrifuge at 4000r/min for 3 min; keep upper clear liquid at -20℃.

B1.2.2 Operational method:

B1.2.2.1 Mark numbers on the back of PVC sensitization membrane before test; add 0.2 ml serum dilution (PBS/T) in each well.

B1.2.2.2 Add test serum and control serum (a negative control and a positive control each batch) according to the number; 10μL each well; mix and stay at 37℃for 5 min (10 min at 25℃).
B1.2.2.3 After incubation, discard diluted serum; wash with NaCl/T for 8 times and leave dry.
B1.2.2.4 Add enzyme-conjugated diluent, 0.2ml each well and stay at 37℃for 5 min.
B1.2.2.5 Discard enzyme-conjugate and wash 8 times; wash again with distilled water and let dry.
B1.2.2.6 Add TMB substrate solution that has been added 3% H_2O_2 (0.2 ml each well); observe the result after 5-10 min.
B1.2.3 Reaction criteria:
Assessment with naked eyes: Judge based on the positive and negative controls. Positive if bright blue is seen and negative if colorless.
Spectrophotometer colorimetric assessment: Not using H2O2 for termination; use 595 nm wavelength colorimetric: positive if P/N >= 2.1(P: optical density of the patient; N: optical density of normal people).

B1.3 Indirect haemagglutination assay (IHA)
B1.3.1 Leishmania promastigote after 10-12 culture is washed with saline; add 4 times of saline and apply ultrasonic treatment for 10s in ice bath at 150 W, 18kc, 300 mA; dilute for 20 times with pH6.4, 0.075 mol/l PBS. Folin method to test protein which is at 200 μg/ml.
B1.3.2 Hydroformylation: Type O red blood cells from healthy people with 10 times of saline are centrifuged for 4-5 times. Add 100 ml of 1% glutaraldehyde for every 8 ml of packed red cells; shake in 4℃water bath for 30 min; hydroformylated red blood cells are washed with saline and distilled water for 5 times; dilute to 15% solution; add 1/10,000 of thiomersal for antiseptic purpose; keep in fridge.
B1.3.3 Sensitization: Dilute hydroformylated red blood cells with pH 7.2, 0.75 mol/l PBS to 1.5%; centrifuge and wash for 3 times; prepare it into 5% cell suspension and add equal amount of 1/10,000 tan-liquor; leave in water bath at 37℃for 30 min; shake every 3-5 min; wash with 5 times as much as the mount of the above-mentioned PBS for 3 times; prepare it into 2.5% red cell suspension with pH 6.4, 0.075 mol/l PBS; add equal amount of pH 6.4 PBS into the suspension to dilute 20 times of the antigens; sensitize in water bath at 37℃for 45 min and shake; centrifuge at 2000 r/min for 3 min; discard upper clear liquid; add pH 7.2 PBS and centrifuge one more time; add pH 7.2 PBS containing 1% normal rabbit serum to prepare into 1% solution.
B1.3.4 Add certain amount of saline onto the dilution plate; add equal amount of test serum in the first well; mix well and double-dilute to 2^{-1}, 2^{-2}…2^{-12} concentration respectively. Take 0.025 ml of serum from each concentration level and add to the 12 6 mm×7 mm wells in the microagglutination plate respectively; add equal amount of sensitized red cells and shake well; put in moisture container and leave at room temperature for 1 hour for assessment with reference to positive and negative controls. Positive if the patient serum agglutinates at 2^{-7} dilution; negative if no agglutination.

B2 Circulating antigen test

B2.1 Monoclonal antibody-antigen spot test (McAb-AST)
B2.1.1 Double-dilute the test serum; drop 2μL onto the nitrocellulose film (0.45μm) and leave at room temperature for 30 min.
B2.1.2 Soak the film into standard buffer solution (0.1mol/l Tris-HCl pH 7.4, 0.25% gelatin, 0.5% NP-40) at room temperature for 2 hours.
B2.1.3 Take the film out and soak in McAb-C-2 (1:100 dilution) at 4℃overnight.
B2.1.4 Wash with buffer solution and incubate with HRP-labelled rabbit anti-mouse IgG (1:1000) at room temperature for 2 hours.
B2.1.5 After wash, put the film in diaminobenzidine substrate solution at room temperature to react for 30 min; wash with distilled water to terminate reactions.
B2.1.6 Observe the dried film; appearance of dark brown or brown spot with clear margin with diameter larger than that of normal serum indicates a positive reaction. No brown spot or similar reaction to that of control serum is seen as negative. For more accurate interpretation, Shimadzu double wavelength TLC scanner (CS-910, S 460nm) can be used to read Integral of surface area (ISA) of the dried film. Positive if the threshold is >1.0; negative if the threshold is <1.0.

B2.2 Enzyme-labelled monoclonal antibody dot-ELISA direct method (McAb dot-ELISA)
B2.2.1 Use glutaraldehyde 2-step method to label purified monoclonal antibodies to prepare HRP-McAb L12F7 and keep at -30℃.
B2.2.2 Double-dilute the test serum repeatedly to 1:8. Drop 2μl of the sample onto nitrocellulose membrane and leave dry at 4℃.
B2.2.3 Soak the nitrocellulose (NC) with serum drops in standard buffer solution (0.1 mol/L Tris-HCl pH 7.4, 0.25% gelatin, 0.5% NP-40) at room temperature; shake and wash 4 time, 10 min each time.
B2.2.4 After wash, add 1:100 diluted HRP-McAb and shake for 2 hours at room temperature; wash with standard buffer solution for 6 times, 10 min each time.
B2.2.5 Add substrate of 4-β-naphthol chloride and shake for 10 min; rinse to terminate the reaction.
B2.2.6 Blue-grey spot indicates positive reactions; no blue-grey spot or only showing serum trace is regarded as negative.
B2.2.7 Both positive and negative controls are needed at each test.

B2.3 Monoclonal antibody enzyme-linked immunoelectronic transfer printing technique (McAb-EITB)
B2.3.1 Treatment of serum sample: take 50μl from each test serum sample and add pH7.4 PBS to 1 ml; centrifuge at 3000 r/min for 30min; add 0.2 ml of 30% PEG (PEG molecular weight 6000) into the upper clear liquid; leave at 4℃overnight; centrifuge at 3000 r/min for 30 min; wash with 50% PEG; centrifuge at 3000 r/min for 30 min; add 50 μl of PBS into the precipitate to dissolve for further use.
B2.3.2 SDS-PAGE technique: Use 10% separating gel (concentrated gel 3.3μl, 3 mol/l

Tris-HCl 1.25 ml, 10% SDS 100 μl, TEMED 5 μl, 10% ammonium persulfate (Ap) 50 μl). 3% concentrated gel (concentrated gel 0.5 ml, 1 mol/l Tris-HCl 0.625 ml, H_2O_2 3.8 ml, 10% SDS 50μl, TEMED 5μl, 10% Ap 25 μl). After polymerization is completed, add 2 μl of serum in each tank. Insert the plate into the electrophoresis tank for electrophoresis at 200V for 50 min.

B2.3.3 Electrophoretic transfer: cover the nitrocellulose membrane with the gel member with alignment and remove air bubbles. Turn on the power; electrophoresis at 200mA, 15V for 30 min.

B2.3.4 After transfer, wash the nitrocellulose membrane with standard buffer solution (Tris-HCl pH7.4, gelatin 0.25%, 0.5NP-40) for 3 times, 10 min each time.

B2.3.5 Add 1:500 diluted HRP-McAb L12H4, 37℃for 1h then 4℃overnight. Wash with standard buffer solution for 3 times; color development in DAB-H_2O_2 system for 10 min; wash with water to terminate reaction.

B2.3.6 Look for specific bands with 130kD, 100kD and 25kD being positive bands.

Annex C Treatment (Supplement)

C1 Anti-leishmaniasis treatment

C1.1 Initial cases: 6-day regimen of Solustilbosan; or 3-week regimen for severe leishmaniasis patients. Solustilbosan should be given slowly intravenously as much as possible. During the 6-day regimen, if the patient develops side effects such as high fever, epistaxis and pain in spleen area, injections can be suspended for a few days until the symptoms improve. The dosage already administered is counted into the total dosage.

C1.2 Uncured cases: Patient still having fever, unchanged WBC count and spleen enlargement with the presence of parasites upon the completion of one course of Solustilbosan treatment should be classified as treatment failure. Dosage of Solustilbosan can be increased by 1/3 to start the 2^{nd} treatment course of an 8-day regimen of 8 injections.

C1.3 Relaspe cases: After treatment, the body temperature is back to normal with general condition and blood counts improved, spleen enlargement reduced and no detection of Leishmania on puncture examination; however, after several months (usually in 6 months), the patient starts to have fever, spleen enlargement again and parasites are found on bone marrow smear. Such cases are classified as relapse cases. Solustilbosan can still be administered but the dosage should be increased by 1/3.

C1.4 Antimony-resistant cases: patients not cured after over 3 treatment courses of antimonials. They are called antimony-resistant leishmaniasis patients who can be treated with the following two aromatic diamidines.

Pentamidine (pintamidine): dissolve the drug in distilled water to get 4% solution for intramuscular injection, 4mg/kg, q.d. for 15 days. Total dosage is 60mg/kg. Local hot compress can be applied to relieve swelling and other reactions around the injection site. If occasionally the patient has low blood pressure, rapid pulse, dizziness and palpitation, inject epinephrine to relieve the symptoms.

Hydroxystilbamidine (hydroxystilbamidine): dissolve the drug in distilled water before use; add 1% procaine solution to make it 2.5%-5% solution for slow intramuscular injection; or dissolve the drug in 50% glucose solution to get 2% solution for intravenous injection, q.d., 2-3mg/kg with the total dosage of 85mg/kg. Aromatic diamidines are not stable in water solution, leading to increased risks of harmful reactions; therefore, only freshly prepared solutions can be used each time.

C2 Symptomatic treatment

C2.1 Anemia: Patients with intermediate anemia should be treated with iron preparation. Patients with severe anemia, in addition to iron preparation, may also be given blood transfusion. Antimonials treatment can be applied after anemia improves.

C2.2 Epistaxis: Wash and clean nasal cavity to locate the bleeding site; apply cotton soaked with 1:1000 epinephrine and 3% ephedrine to the bleeding site, or use gelatin sponge to cover the bleeding site.

C3 General treatment

Patient on treatment should rest and prevent flu, and be supported with nutritious and high energy food such as eggs, liver, bean curd as well as sufficient multiple vitamins to accelerate recovery.

C4 Management of complications
C4.1 Pneumonia: Patients complicated with pneumonia should not be given antimonials or aromatic diamidines. If pneumonia occurs during the course of the anti-leishmaniasis treatment, the injection of drugs should be stopped immediately and antibiotic treatment should be started. After pneumonia symptoms are gone, anti-leishmaniasis drug treatment can be resumed.
C4.2 Acute agranulocytosis: Penicillin treatment should be started immediately to prevent secondary infection. If it occurs during the course of antimonials treatment, anti-leishmaniasis drugs should be stopped immediately until the symptoms go away before resuming antimonial or aromatic diamidine drug treatment. In some cases, leishmaniasis may also lead to this problem and it has nothing to do with antimonials. In such cases, the administration of antimonials is not only harmless but also helps to increase granulocytes as the patient improves.
C4.3 Gangrenous stomatitis: Penicillin treatment should be given as early as possible while continuing the antimonial treatment.
C4.4 For patients with severe heart, liver or kidney problems, antimonials should be used with great caution while giving symptomatic treatment.

Additional notes:
These criteria are proposed by the Ministry of Health of the P.R.China.
These criteria are drafted by the Institute of Parasitology, Chinese Academy of Preventive Medicine.
The drafters are Qu Jingqi and Guan Liren.
The Infectious Disease Supervision and Management Office is authorized by the Ministry of Health to interpret these criteria.
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