CYPRESS DIAGNOSTICS

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96 Test

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Leishmania Ab

Leishmania Ab.

Leishmania Ab is an enzyme immunoassay for the detection of antibodies against Leishmania (species donovani, major, mexicana, braziliensis) in human serum.

General Information

Leishmaniasis is a parasitic disease caused by different species of a flagellate protozoa genus Leishmania. The most characteristic clinical forms of the disease are:

a) Visceral Leishmaniasis or Kala-azar.

This is produced by L. donovani donovani (India, Africa), L. donovani infantum (Mediterranean zone, Asia, Middle East) and L. donovani chagasi (South America).

The phagocytosed parasites are numerous in the reticuloendothelial (RE) cells of the spleen, liver, lymph nodes, bone marrow, intestinal mucosa and other organs. These organs tend to enlarge due to the enormous grow of the RE cells. Incubation periods last 1 to 4 months.

The initial fever is intermittent and can rise toe 40°C. A marked leucopenia with relative monocytosis, anemia and thrombocytopenia develop, rendering the patient especially susceptible to secondary infections.

Untreated disease usually progresses to fatal termination within 2 years, although fulminating infections may cause death within few weeks.

b) Cutaneous Leishmaniasis.

This is produced by L. major or tropicana (Old World) and by a complex of L. mexicana and braziliensis (America). The disease is known with different names in each country.

In humans the disease is limited to the cutaneous tissues and occasionally to the mucous membranes. Incubation periods last from 2 weeks to several months.

It is the least severe form of the disease.

c) Mucocutaneous or American Leishmaniasis.

Mainly produced by L. braziliensis braziliensis, although other species produced similar mucocutaneous lesions.

The clinical appearance and histopathology of american leishmaniasis are identical to cutaneous leishmaniasis, except that it may produce later mucous membrane involvement. The mucosal lesions are painful and can cause great deformity with erosion of the nasal septum, palate or larynx.

The life cycle involves an alternate existence in a vertebrate and an insect host.

The natural host, besides humans, includes domestic dog and a variety of wild mammals such as desert or forest rodents and sloths.

The invertebrate hosts are sand flies of the genus Phlebotomus, renamed Lutzomya in America. After parasites within infected cells are ingested with the blood meal, they transform into flagellates and multiply in the gut of the insect. In 8 to 20 days, the anterior gut and pharynx are partially blocked by flagellates. When the sand fly attempts a subsequent blood meal, some of the infective promastigotes are dislodged and introduced into the skin. Transmission can also occur by contamination of the bite wound and by contact.

Leishmaniasis has a worldwide distribution, mainly in tropic and sub tropic zones.

The incidence of the disease is rising and infection is calculated to affect some millions of people.

It is one of the six most important infectious diseases in the world.

Principle of the test

The Cypress Diagnostics' Leishmania Ab semi - quantitative test is based on the principle of an enzyme Immunoassay.

On the surface of the microtiter wells, inactivated soluble antigens of Leishmania are bound. Diluted serum samples, controls and calibrator are pipetted into the wells and incubated at room temperature. Present antibodies bind to the immobilized antigens. Unbound material is removed in a washing step.

In a second step, Anti-human Ig-conjugate (peroxidase-conjugated IgG antibody) is added, which binds to the antibodies bound before.

After incubation, unbound conjugate is removed by washing. Substrate (urea peroxide) and chromogen (TMB) are added to the wells and incubated at room temperature.

The enzyme bound in the wells converts the colorless substrate/chromogen to a blue color. Addition of stop solution converts the color from blue to yellow.

The absorption is measured at a 450 nm wavelength (optional: reference wavelength >620 nm).

Precautions

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results :

1. Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).

2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.

3. Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.

4. Discard the internal transparent stoppers, that come in the vials of some of the reagents (Sample Diluent, Wash Solution, etc.) once these vials are opened for the first time. The purpose of this stopper is to avoid leakage during transport.

5 Once the assay has been started, all subsequent steps should be completed without interruption and within the recommend time limits.

6. Use a separate disposable tip for each specimen to prevent cross-contamination.

7. Do not use or mix components from different lots.

8. Do not use reagents after expiration date.

9. It is recommended to dispense in a clean test tube the amount of reagent needed for each run in order to prevent possible contamination of the vials content (specially the Substrate A and B solutions). Clean the micropipettes after each reagent-dispensing step.

10 All specimens and controls should be run at the same time so that all testing conditions remain the same.

11. The quality control specimens (controls) should be run every time samples are assayed to check the test performance.

12. Check both precision and accuracy of the laboratory equipment used during the procedure (micropipettes, ELISA reader, etc.).

The Health and Safety instructions listed below must be followed :

1. Do not smoke, eat, drink or pipet by mouth in the laboratory.

2. Wear disposable gloves whenever handling patient specimens.

3. Use a 5% sodium hypochlorite solution to wipe up spills.

4. Treat all materials used in the test as if they were infectious. Autoclave all materials for 1 hour at 121,5°C. Add sodium hypochlorite to liquid waste and disposable material in order to reach a final concentration of 5%. Leave the material at least 30 minutes in this solution. Incinerate all disposable material.

5. All serum samples must be considered as potential infectious and must be handled with usual precautions.

6. The positive and negative controls have been tested and found no reactive to HBsAg, HIV and other infectious agents. As no available test methods can offer, presently, complete assurance that these agents are absent from the sample tested, all the controls must be regarded as potentially infectious and must be handled with usual precautions.

7. Avoid contact of substrate and stop reaction solutions with skin and mucosa (possible irritation, burn or toxicity hazardous). In case of contact, rinse the affected zone with plenty of water.

8. Handling and disposal of chemical products must be done according to good laboratory practices (GLP).

Material provided

The reagents in one kit are sufficient for 96 determinations.

Each test kit contains :

1 x 12 Microwell Strips (breakable) with 8 wells
each in a frame; coated with inactivated Leishmania antigen in a resealable foil bag.

1 x Sample Buffer (50 ml, pH 7.4), ready to use, dyed yellow, colorless lid

1 x Washing Buffer (50 ml, ph 7.4), 20x conc., dyed blue, brown lid

1 x Calibrator (1.2 ml) green screw cap.

Inactivated human serum, ready to use.

1 x Positive Control (1.2 ml), red lid;
inactivated human serum, ready to use

1 x Negative Control (1.2 ml), colorless lid;
inactivated human serum, ready to use

1 x Anti-human Ig-Conjugate (12 ml), red lid;
HRP-Conjugated IgG-antibody (goat), ready to use

1 x Substrate (6 ml), green lid;
urea peroxide, ready to use

1 x Chromogen (6 ml), blue lid;
tetramethylbenzidine (TMB), ready to use

1 x Stop Solution (6 ml), yellow lid;
1 M sulfuric acid

1 x Insert

Material needed but NOT provided

Distilled or deionized water

Test tubes

Vortex mixer

Micropipettes for volumes of 10 - 100 µl and 100 - 1000 µl

Measuring cylinder (1000 ml)

Microplate washer or multichannel pipette

Microplate reader (450 nm,)

Absorbent paper

Stability

Reagents must be stored at temperatures between 2°C and 8°C.

Expiration date is the last day of the month of the expiration date found on the box, vials and microstrips labels.

Microbial contamination has to be avoided.

Exposure of the reagents to ambient temperatures for short periods does not affect the stability of the reagents.

The diluted washing buffer has a shelf life of 4 weeks if stored at 2°-8°C.

Allow reagents and microwell strips to get to room temperature before use. To avoid moisture within the strips, do not take the strips out of the foil bag before having reached room temperature. The foil bag should be opened with a pair of scissors without detaching the fastener. Return any unused strips to the foil bag, reseal and store them directly

at 2°-8°C.

The colorless chromogen must be protected from exposure to direct light to avoid deterioration or coloration by oxidation. If the chromogen has turned blue, the reagent should be discarded.

DO NOT FREEZE ANY OF THE REAGENTS. Freezing can cause irreversible damage to the reagents.

The following criteria may indicate a reagent deterioration :

- A turbidity or a blue coloration of the colorless chromogen prior to its use.
- An absorbance value (O.D.) of the negative control at 450 nm > 0.3
- An absorbance value (O.D.) of the positive control at 450 nm >0.6

Serum Specimens Information

Fresh serum is required. Serum may be stored at 2°-8°C for op to seven days. If the test cannot be carried out immediately, store at -20°C (avoid repeat freezing and thawing of samples, it may cause false results).

If they have been stored at 2-8°C, diluted samples can be used for up to 7 hours.

Application of heat treated, lipemic, hemolytic, icteric or tubid samples can lead to wrong results.

Allow the sample to stand till the blood has coagulated. Centrifugation can be used to separate the serum from the rest of the components.

Test Procedure

Preparation

Bring all reagents and the microwell strips for at least 30 minutes to room temperature before use. Mix the reagents well before use. Reproducibility in any EIA depends on exact pipetting, the observance of incubation times and temperature and the consistency of wash sequences.

During the washing steps, take care that all wells are filled with buffer and that the liquid is completely removed from the wells. Do not allow microwells to dry between steps.

Avoid direct sunlight during all incubations. Covering the microtiter plate is recommended.

Except the washing buffer, all reagents are ready to use.

Preparation of the washing buffer

One (1) part of the concentrated washing buffer is diluted with 19 parts of distilled water. Crystals in the buffer concentrate can be dissolved in a water bath at 37°C.

Add 50 ml of the concentrated washing buffer to a 1000 ml graduated cylinder. Bring the final volume to 1000 ml with distilled or deionized water. The diluted washing buffer has a shelf life of 4 weeks if stored at 2-8°C

1. Preparation of the samples

Before starting the test, serum samples have to be diluted 1 : 20 with the sample buffer.

e.g. 10 µl serum + 190 µl sample buffer

The controls included in the kit are ready to use and must not be diluted.

2. After insertion of a sufficient number of cavities into the microwell holder, 100µl of the positive control, the calibrator, the negative control and the diluted sera are pipetted into the wells and incubated for 10 min at room temperature. It is recommended to use the calibrator in double determination in each run.

3. Washing

Decant or aspirate all microwells into a waste container with a disinfectant. Ensure complete removal of the liquid from the microwells by tapping the inverted plate onto absorbent paper. Then wash all wells 5 times with 300 µl of prepared washing buffer. Be sure to remove residual washing solution by firmly tapping the inverted microwells on absorbent paper after single washing steps.

If a microplate washer is used, take care that the washer is adjusted to the used microplate type.

4. Second incubation

Add 100µl or 2 drops of anti-human Ig-conjugate to all wells. Incubate the plate for 5 min at room temperature.

5. Washing

Wash 5 times according to step 3

6. Third incubation

Add 50µl or 1 drop of substrate and 50 µl or 1 drop or chromogen into each well. Incubate the plate for 10 min at room temperature in the dark. Following the incubation, the reaction is stopped by adding 50 µl or 1 drop of stop solution to each well. After careful mixing (soft tapping on the edge of the plate) the absorbance is measured in a microplate reader at 450 nm (optional: reference wavelength > 620 nm).

Remark

Highly positive patient samples can cause dark precipitates of the chromogen.

Quality Control

For the quality control, positive control, calibrator (double determination) and negative control must be carried along with each test run.

The test was carried out correctly, if the positive control shows an absorbance value (O.D.) at 450 nm greater than 0,6.

The ratio between the O.D. of the positive control and the O.D. of the calibrator must be greater than 1,5.

The absorbance value of the negative control at 450 nm must show a value lower than 0,3. Additionally, the O.D. of the negative control must be less than the O.D. of the calibrator

Calculation of Results

a. Calculate the average O.D. of the calibrator. This value is the cut-off value of the assay.

b. Divide the sample O.D. by the value obtained in point a.

e.g. calibrator well 1: O.D. = 0.541
calibrator well 2: O.D. = 0.554
Sample = 1.441

Cut off value = $\frac{0.541+0.554}{2} = 0.548$

Sample ratio = $\frac{1.441}{0.548} = 2.63$

Interpret the results using this sample ratio as follows:

sample ratio < 0.9 = negative result
0.9 < sample ratio < 1.1 = equivocal result
1.1 < sample ratio < 1.5 = low positive result
1.5 < sample ratio < 2.5 = medium positive result
2.5 < sample ratio = high positive control

Equivocal and low positive results can happen in these cases :

- Persons that were bitten by infected Phlebotomus at endemic areas and did not develop the disease but have a low antibody level.
- Persons that presently have or suffered from asymptomatic disease, so they still have a low antibody level.
- Persons that suffered from the disease in the past and still have a low antibody level.
- Persons that are developing the disease.
- Unidentified and unspecified reaction factors.

These cases should be further investigated, retesting a fresh new sample and including clinical symptoms and additional tests, to know at what group they belong. In case the same result is obtained, the test must be repeated with a new sample after 2-4 weeks.

Middle and high positive results can happen in persons that are suffering from the disease or have suffered a recent infection, or with past infections that still have a high antibody level. It is necessary to compare these results with clinical symptoms and additional tests.

Negative antibody findings cannot exclude a leishmaniasis. Due to low antibody titer at early time of an infection, the test can show negative results. If a clinical suspicion subsists, after two to four weeks another patient' sample should be tested.

It has been found that in South America, this test may cross react in some occasions with serum positive to T. cruzi (or Chagas disease). Therefore, special caution must be taken in areas of South America where Chagas disease is present, when using this test.

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QUICK REFERENCE TEST PROCEDURE

1. Bring all reagents to room temperature.

2. Dilute the washing Buffer.

3. Dilute the serum samples.

4. Pipet 100µl of the positive control, the calibrator (double determination), the negative control and the diluted samples into the microwells; 10 minutes incubation at room temperature

5. Discard the incubate and wash 5 times with 300 µl washing buffer

6. Add 100 µl or 2 drops of anti-human Ig-conjugate; 5 minutes incubation at room temperature

7. Discard the incubate and wash 5 times with 300 µl washing buffer

8. Add 50 µl or 1 drop each of substrate and chromogen; 10 min incubation at room temperature in the dark

9. After addition of 50 µl or 1 drop stop solution spectophotometric determination at 450 nm (optional: reference wavelength > 620).

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