

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 2×10^8 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.

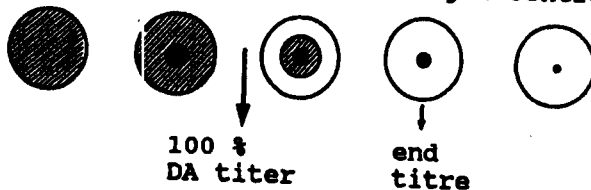
3. For a 12-row microtitre plate pipette 50 μ l of diluent to all wells except no 2.
4. Into well 2 pipette 100 μ l of the 1/100 dilution of the serum under test (see 1).
5. Transfer 50 μ l from well 2 to well 3, mix then transfer 50 μ l from well 3 to well 4. Continue this operation across the plate, discarding the 50 μ l 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 μ l into well 1 (no serum control). Next pipette 50 μ l antigen to well 12, 50 μ l 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 anticlockwise 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



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.