IMMUNOHISTOCHEMICAL TECHNIQUE FOR DETECTING M. bovis IN FORMALIN-FIXED TISSUES OR CRYOSTAT SECTIONS

Types of Tissues: Lungs
Trachea explants
Impression smears/ Cell monolayer
Joint capsules & tendon sheath
Mammary gland, liver, kidney, gut, etc.

Fixation:

Formalin- recommended, as antigen better stained
- 10% buffered, neutral
- 10 to 18 hrs, e.g. O/N; then transfer into 70% ethanol and process as soon as possible.
- 10 to 15 mins for impression smears / cell monolayer
- 1 to 2 hrs for joint capsules or tendon sheath

Cryostat sections in O.C.T. Compound(Tissue-Tek)

- fix freshly cut sections in acetone for 7 minutes
- freeze at -70 C until used.

Formalin-fixed tissues
- submitted in 70 % ethanol
- 95% ethanol, 1 hr x 2 stations
- 100% ethanol, 1 hr x 3 stations
- Xylene, 1 hr x 3 stations

Paraffin- embedding:

- sectioning at 4 microns and mounting on 3-NH2propyl-triethoxy-silane-coated slides or other adherent slide types (SuperFrost® Plus, etc).

De-paraffinization:

Station 1- Place slides on slide warmer at 55C for 30 min
Station 2- Dip in xylene and leave there 5 min
Station 3- Dip in xylene and leave there 5 min
Station 4- 10 dips in 100% ethanol
Station 5- 10 dips in 100% ethanol
Station 6- 10 dips in 95% ethanol
Station 7- 10 dips in 95% ethanol
Station 8- 10 dips in 80% ethanol
Station 9- 10 dips in 70% ethanol
Station 10- 10 dips in distilled water
Station 11- 10 dips in distilled water

It is best to not reuse these reagents. Xylene stations can be used for up to 25 slides.

Proceed to next stage without drying!

Immunoperoxidase staining:

1.Treat slides with 1% hydrogen peroxide in methanol (13 ml 30% H_2O_2 in 400 ml methanol), for 30 min at RT to inactivate endogenous peroxidase, and wash for 10 mins in distilled water.

Omit step 2 for cryostat sections. But rinse 3X in PBS, 3 min each, to remove OCT.

2. Microwave treatment; have at hand 400 ml ice-cold TBS (10X TBS is 1.5 M NaCl in1 M Tris pH 7.5)

2a. Change slides to plastic basket, without drying! Place slides in citrate buffer pH 6.0 (in a 2 L glass beaker, add 9 ml 0.1 M citric acid, 41 ml 0.1 M sodium citrate, 450 ml dH_2O) and microwave for 2 min at setting that gives 80-90C (2.00; Power Level 5 times; Start)

2b. Cool slides rapidly in ice-cold TBS

2c. Wash in TBS at room temperature. Mycoplasma antigens are better retrieved on slides with this protocol. [0.1 M citric acid is made with 10.5 g citric acid monohydrate in 500 ml dH_2O]. [0.1 M sodium citrate is made with 14.7 g sodium citrate in 500 ml dH_2O].

3. Transfer individual WET slides into slide chambers (Shandon), ensuring no air bubble is trapped.

4. **Do an avidin-biotin block.** Alternatively, detection with Chemicon's poly-HRP Goat anti-mouse reagents makes avidin-biotin unnecessary since the poly-HRP secondary provides even more amplification than traditional ABC biotin systems.

4a. Apply 2 drops of reagent A (avidin-biotin block kit- Zymed) per slide chamber.

4b. Incubate for 10 minutes at room temperature

4c. Fill chambers with TBS for 5 minutes, twice.

4d. Apply 2 drops of reagent B (avidin-biotin block kit- Zymed) per slide chamber.

4e. Incubate for 10 minutes at room temperature

4f. Fill chambers with TBS for 5 minutes, twice.

5. Apply 4 drops of primary antibody (rabbit x M. bovis 1:400) O/N at 4 C. Monoclonal concentrated supernates at 1:400 dilution or ascites at 1:1000. One slide should be the TBS control. We recommend a pool of MAB970 and MAB9701 (Chemicon) with both reagents at final dilution of 1:1000. This assures detection of all strains of M. bovis.

6. Rinse slides with 0.05 M Tris buffer, pH 7.6, for 5 mins.

7. Apply 0.08 mg/ml globulins (or 1% normal serum) from the species in which the secondary antibody is made, for 5 minutes - 4 drops per slide. Use 0.08 mg/ml goat globulins when using DAKO LSAB kit - yellow bottle.

8. Apply biotinylated goat anti-mouse secondary antibody for 30 minutes at RT - 4 drops per slide (DAKO LSAB 2 kit - yellow bottle). Apply biotinylated goat anti-horse secondary antibody (Vector Labs) for polyclonal controls if using horse anti-M. bovis polyclonal.

9. Fill all slide chambers with 0.05M Tris for 5 mins, i.e. wash x1.

10. Apply peroxidase conjugated streptavidin for 30 mins-4 drops per slide. (DAKO LSAB kit 2- red bottle.

11. Repeat washing step 9.

12. Make DAB solution fresh. Follow instruction for re-constitution. Apply 4 drops per slide for 2 minutes. DAB reagents tend to get old before the expiration date, so replace them as needed. Extend (up to 2X) incubation time as reagent nears expiration date. DAB solution can also be made using PBS with 1 µl/ml 30% H_2O_2 as buffer, adding the stock DAB to this at same proportion (1 drop/ml buffer). Filter through filter paper just before use.

13. Fill all slide chambers with distilled water for 5 mins, twice.

14. Re-assemble slides on slide rack as fast as possible (avoid drying), and dip rack in distilled water to rinse off excess reagents thoroughly.

15. Counterstain with hematoxylin (filter into jar through glass fiber filter) for 90 secs. Save stain for re-use up to 5 times.

16. Rinse slides in PBS to allow blueing of the counterstain.

17. Dehydrate : 10 dips in 95% ethanol, 1x
10 dips in 100% ethanol, 2x
10 dips in Hemo-De (Fisher), 1x
10 dips in Pro-par, 2x

18. Leave slides in Pro-par, and mount with 1 drop of Shandon mountant which mixes well with Pro-par. Let mounted slides dry O/N without touching covers.

References:

1. Schultheiss, P. C., Collins, J.K., and Carman, J. 1993. J. Vet. Diagn. Invest. 5: 12-15.

2. Adegboye, D. S., Rasberry, U., Halbur, P. G., Andrews, J. J., and Rosenbusch, R. F. 1994. Monoclonal antibody-based immunohistochemical technique for the detection of Mycoplasma bovis in formain-fixed paraffin-embeded calf lung tissues. J. Vet. Diagn. Invest. 7:261-265.