Laboratory Techniques and Procedures

AFB-Smear preparation/ fluorescent staining of smears

According to Padma *et al.* (87) the smear positivity rate of fluorescent stain is higher than ZN stain. The procedures of the test described as follows:

1. Thoroughly one side of a clean glass slide was flamed to be used for the smear and let cooled down before smearing.
2. A smear was prepared from the specimen on the flamed side of the slide and allowed to air dry.
3. The smear was fixed by passing the slide through a flame and flooded the slide with Phenolic Auramine Solution.
4. The stain was allowed to remain on surface of the slide for 15 minutes without drying.
5. Rinsed with distilled water and shaked off excess liquid.
6. The slide was flooded with decolorizer (Acid Alcohol Solution) for 2-3 min.
7. Rinsed thoroughly with distilled water and shaked off excess.
8. Then the slide was flooded with counterstain (Potassium permanganate Solution) 3-4 min and the slide was not allow to dry at this time.
9. After that it was rinsed with distilled water and allowed to air dry.
10. Examine microscopically under a fluorescent microscope (UV light source) at low and high magnification.
11. Slides was screened on high power (400X) and verified under oil immersion lens.
12. Slides of Acid Fast Bacilli was stained individually to prevent Acid Fast Bacilli carry-over from slide to another. This is a part of identification.

Interpretation of results: Acid Fast Bacilli: luminous yellow rods in dark field.

Note; the procedure is adapted from manufacturer guideline; Sigma-Aldrich website available at www.sigmaaldrich.com.

Comparative Intradermal Tuberculin Test

This test was conducted as used by Alemayehu *et al.(48)*. In short, within a few weeks of the identification of a human TB case, the cattle owned by the patient was identified and tested for TB with the comparative intradermal tuberculin test. A study team including a

veterinarian and laboratory technicians administered the intradermal comparative tuberculin test, interviewed farmers, and collected milk samples from reactive cows in the herds. Cattle from both households (cattle from human TB cases and cattle from the control group) above 6 months of age was tested by comparative intradermal tuberculin test. During testing parameters such as age, sex, body condition score was recorded for each of the study cattle. Avian purified protein derivative (PPD) was used to exclude mycobacteria which are not members of Mycobacterium tuberculosis complex. Two sites on the skin of the mid neck of the study animals, 12 cm apart, was shaved and skin thickness was measured in millimeters with digital calipers before the injection of tuberculin. Aliquots of 0.1 mL of 20,000 IU/mL bovine PPD and 0.1 mL of 25,000 IU/mL avian PPD was injected into the dermis at these sites. A correct injection was confirmed by palpating a small pea-like swelling at each site of injection. After 72 hours, the thickness of the skin at the injection sites was again measured. The interpretation of the result was made on the basis of the difference in skin indurations at bovine PPD (B) and avian PPD (A) injection sites. A difference (B - A) of 4 mm or above was considered as a positive while a difference of less than 2 mm will be considered as a negative tuberculin test. Readings of a difference between 2 and 4 mm was considered inconclusive /doubtful.

Milk sample collection and culture

Approximately 30 mL of the last few streams of milk was collected by the owners at milking into sterile universal bottles from each quarter of TB-positive dairy animals. Milk samples was cultured according to Kazwala *et al.* (16). Briefly, samples was centrifuged at 1310 g for 15 min and the supernatant discarded. The sediments was suspended in 2 mL of sterile physiological saline solution and decontaminated with equal volume of sterilized 4% NaOH solution. One or two drops of 0.05% phenol red indicator was added and then neutralized using concentrated HCl. The suspension was centrifuged at 1310 g for 15 min at 4 C and the sediment inoculated onto two slants of Lowenstein-Jensen media as indicated above. Growth for *Mycobacteria* was checked every week. Positive cultures was sub cultured onto another set of media and incubated for another 3–4 weeks for further identification (15). Initial identification of *Mycobacterial* species from animal tissue

was based on the rate of growth, pigment production, and colony morphology as described in OIE (2009)(88). When visible colonies are observed, AFB staining was performed to confirm the presence of acid-fast bacilli (89).

Culturing of Sputum

Sputum samples was collected in sterile universal tubes from smear positive pulmonary TB patients. The sputum samples was processed (decontaminated and neutralized) for mycobacterial culturing according the standard operating procedure described earlier by WHO (2008). Thereafter, 100 ml neutralized sample was inoculated onto two slants of Lowenstein-Jensen (LJ) media, one supplemented with pyruvate and one with glycerol, and incubated at 37 ℃ for up to 5–8 weeks. Growth of mycobacteria was monitored every week for up to 8 weeks. Sample negative for AFB after 8 weeks of growth was considered negative.

SD Bioline TB Ag MPT64® test

SD MPT64 Rapid test was performed according to the manufacturer's guidelines. Three or four colonies of mycobacterial strains grown in Löwenstein-Jensen were emulsified in 200 μL of extraction buffer. Next, 100 μL were placed in sample wells of the test and the results were visually assessed based on color development after incubating at room temperature for 15 min(90).Positive result was indicated by the presence of only two color band (one control band and one test band). The presence of only one control band within the result window indicated a negative result. Faint color band was recorded as a weak positive and the sample retested(91).The SD BIOLINE TB Ag MPT64 RAPID® test is a simple and rapid assay using a mouse monoclonal anti-MPT64 antibody that is able to discriminate between MTC and NTM by immune-chromatography. The use of this kit was based on the detection by chromatographic diffusion of a specific MPT64 antigen of MTC (92).