

ATTACHMENT VI

AUSTRALIAN
STANDARD
DIAGNOSTIC
TECHNIQUES
FOR ANIMAL
DISEASES

STANDING
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Bovine Tuberculosis

Immunodiagnosis

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Bovine Tuberculosis

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1. Introduction

The National Brucellosis and Tuberculosis Eradication Campaign (BTEC) began in 1970 with the aim of eradicating these two diseases from Australian cattle. Australia was officially declared free from bovine brucellosis in 1989 and impending free from bovine tuberculosis in December 1992. Monitoring of the national herd for *Mycobacterium bovis* infection will have to be maintained until at least the year 2008.

The standard method for detection of tuberculosis in cattle is the tuberculin test, which involves the intradermal injection of tuberculin (purified protein derivative; PPD) and the subsequent detection of swelling at the site of injection three days later. The comparative intradermal tuberculin test is used to differentiate between cattle infected with *M. bovis* and those sensitised to tuberculin due to exposure to other mycobacteria. This sensitisation can be attributed to the large antigenic cross-reactivity between mycobacterial species. The comparative tuberculin test is employed in herds where there is the possibility that cattle are infected with, or sensitised to, mycobacteria other than *M. bovis*. It involves the injection of bovine PPD and avian PPD into different sites on the neck and measuring the responses three days later. An animal in which the predominant response is to bovine PPD is deemed to be a positive reactor; i.e. sensitised by infection with *M. bovis*. Recently, a whole blood culture and interferon- γ (IFN- γ) enzyme immunoassay (EIA) system for the detection of tuberculosis in cattle was developed (Rothel *et al.*, 1990). This assay is similar to the comparative intradermal test in that the animal's responses to both bovine PPD and avian PPD are measured. In a field trial involving over 12,000 cattle, the IFN- γ assay showed a sensitivity of up to 93.6% compared to that of 65.6% for the single intradermal tuberculin test (Wood *et al.*, 1991). The specificity of the IFN- γ assay varied between 96.2 and 98.1% depending on the method of interpretation. A maximum sensitivity of 95.2% was obtained by using the IFN- γ assay and the caudal fold test in combination. The IFN- γ assay for the diagnosis of bovine tuberculosis has been accredited for use in Australia by the Standing Committee on Agriculture.

2. Single Intradermal Caudal Fold Tuberculin Test

In cattle the intradermal tuberculin test (skin test) is usually performed in the caudal fold of the tail, but can be performed in the skin of the neck if desired. The test involves the intra-dermal injection of bovine PPD tuberculin (0.3 mg) and examination of the site of injection 72 hours later.

2.1. Technique

A syringe capable of accurately delivering 0.1 mL (usually a calibrated multidose McIntock syringe) and short needles (3–4 mm) of 26 gauge or finer should be used for injections. McIntock syringes should be regularly calibrated. Syringes and needles should be carefully examined before and during use to ensure that they are working efficiently. They should be cleaned and then sterilised by boiling before use. It is important that syringes and needles are free from disinfectant or antiseptics as even slight traces of these may damage the tuberculin and invalidate the test or cause inflammation resulting in a false positive response. Dental type syringes should not be used as they may lead to the inaccurate delivery of tuberculin.

Bovine tuberculin PPD (3 mg or 150 000 units/mL) is obtained from CSL [45 Poplar Road, Parkville, Vic. 3052, Australia; Tel. (03) 389 1911, (008) 032 675; Fax (03) 389 1686] and is supplied sterile. It has been standardised to contain biological activity equivalent to the stated concentration in mg/mL of the Weybridge Reference Standard Preparation of Bovine Tuberculin PPD. Bovine tuberculin PPD from suppliers other than CSL Ltd may be used as long as the biological activity has been similarly standardised. PPD must be stored at 2–8°C in the dark. PPD should not be used after the day on which the container is opened.

The injection site for the caudal fold skin test is 6–10 cm from the base of the tail, at the junction of hair-bearing and hair-free skin. Before injection, the site of injection should be wiped thoroughly clean. Cattle can be injected in the left or right caudal fold, but all cattle in a particular test group should be injected on the same side. If skin testing in the neck, the injection site should be situated at the border of the anterior and middle thirds of one side of the neck at least 10 cm below the crest. A 3–4 cm diameter area should be clipped free from hair and PPD injected into the centre of this area.

Bovine tuberculin PPD (0.1 mL) is injected intradermally in the caudal fold or neck. The needle, bevel edge outwards, is inserted obliquely into the dermis such that the needle point will reach the deeper layers of skin without penetrating into the subcutaneous tissue. A correct injection is indicated by the raising of a small pea-like swelling ('bleb') at the site of injection. If there is any doubt as to the correct injection of the tuberculin in the dermis, that is, no 'bleb' is raised, a further injection should be made.

2.2. Interpretation

The site of injection of tuberculin is examined, both visually and by palpation, in all cattle approximately 72 hours after injection. A positive reaction is indicated by any visible or palpable swelling at the site of injection.

2.2.1. Reactor

An animal with a positive reaction in a herd with a history of tuberculosis or an inadequate history.

2.2.2. Suspect

An animal with a positive reaction in a herd where the history is not suggestive of tuberculosis, or which has a history of non-specific sensitisation.

The significance of a suspect reactor will depend on the herd history and on the policy of the Department of Agriculture in the relevant State or Territory.

3. Comparative Intradermal Tuberculin Test

The single intradermal comparative tuberculin test (comparative test) is carried out using both avian and bovine tuberculin PPDs. The tuberculins are injected intradermally at different sites in the neck of cattle and responses measured 72 hours later.

3.1. Technique

Two syringes of the type detailed in 2.1. are required for this assay. Syringes should be clearly marked to distinguish those used for avian PPD from those used for bovine PPD.

Bovine tuberculin PPD (1 mg or 50 000 units/mL) and avian tuberculin PPD (25 000 Units/mL) are obtained sterile from CSL Ltd. Bovine tuberculin PPD and avian tuberculin PPD have been standardised to contain biological activity equivalent to the stated concentration of the Weybridge Reference Standard Preparation of Bovine Tuberculin PPD and the International Standard Avian Tuberculin PPD, respectively. Tuberculin PPDs from suppliers other than CSL Ltd may be used as long as their biological activity has been similarly standardised. Tuberculins must be stored at 2–8°C in a dark place and care should be taken to ensure that they are not subjected to extreme temperatures. Tuberculins should not be used after the day on which the container is opened. UNDER NO CIRCUMSTANCES should bovine tuberculin PPD 3 mg/mL be diluted for use in the comparative test.

The injection sites should be situated at the border of the anterior and middle thirds of one side of the neck; the upper site should be at least 10 cm below the crest and the lower site should be approximately 12 cm from the other on a line roughly parallel with the line of the shoulder. The upper site should be used for bovine PPD and the lower for avian PPD. A 3–4 cm diameter area should be clipped free from hair at each site and if necessary cleansed with water before the tuberculins are injected. All cattle must be individually identifiable either by ear tagging, tail tagging or tattooing.

Prior to the injection of tuberculin, the skin thickness at the site of injection is measured. A fold of skin at the centre of each clipped area should be taken between the forefinger and thumb, its thickness measured with calipers graduated in millimeters (Vernier type or similar) and recorded next to the animals identification number. The thickness of the skin fold at the site of injection must be measured before injecting the tuberculin, as the measurement of a skin fold at an adjacent site after the reaction has developed would lead to faulty interpretation. The tuberculins (0.1 mL) are injected intradermally into the centre of the clipped areas of the appropriate sites as detailed in 2.1. If there is any doubt as to the correct injection of the tuberculin in the dermis, that is, no 'bleb' is raised, a further injection should be made preferably on the other side of the neck, but in a similar site, and this fact should be recorded.

The skin fold thickness of each injection site should be measured with calipers 72 hours after injection and recorded next to the animals identification number. Measurements must be taken carefully as a difference of even 1 mm may determine whether an animal is positive, suspect or negative. The measurement must be taken across the entire breadth of the swelling, i.e. the swelling must be at the apex of the skin fold when picked up for measurement.

3.2. Interpretation

There are two methods of interpreting the comparative test, the *Standard Interpretation* and the *Severe Interpretation*.

3.2.1. Standard Interpretation

An animal is considered to be a POSITIVE reactor if the increase in the skin thickness at the bovine PPD site exceeds 4 mm and is greater than 4 mm above the increase at the avian PPD site. An animal is deemed to be a SUSPECT reactor if the increase in the skin thickness at the bovine PPD site is greater than 2 mm and between 1 and 4 mm greater than the increase at the avian PPD site. All other results are considered negative.

3.2.2. Severe Interpretation

An animal is considered to be a POSITIVE reactor if the increase in the skin thickness at the bovine PPD site is greater than 2 mm and at least 3 mm greater than the increase at the avian PPD site. An animal is deemed a SUSPECT reactor if the increase in the skin thickness at the bovine PPD site is greater than 2 mm and differs from the increase at the avian PPD site by less than 3 mm. The exception to the above is if the increase in the skin thickness at the bovine PPD site is either 3 or 4 mm and the increase at the avian PPD site is less than 3 mm. In this instance the animal is judged to be POSITIVE. All other results are considered negative.

Fig. 1 explains the two methods of interpretation. The method of interpretation that is used for a particular herd will depend on the herd history and on the policy of the Department of Agriculture in the relevant State or Territory.

4. Interferon- γ Assay

The interferon- γ (IFN- γ) assay system has been developed as a convenient and sensitive alternative to the intradermal tuberculin test. The test is based on the premise that only cattle infected with *M. bovis* will have circulating T lymphocytes that will react to bovine PPD. In a simple whole blood culture system, these sensitised T lymphocytes respond to the presence of bovine PPD by secreting IFN- γ which is then quantified in the plasma by an EIA specific for bovine IFN- γ .

The assay system involves the incubation of aliquots of heparinised blood with bovine PPD, avian PPD (cross-reactive antigen) and phosphate buffered saline (PBS, pH 7.3; negative control) for 24 hours at 37°C. Initially, only plasma from the bovine PPD samples is harvested and assayed for the presence of IFN- γ in the EIA. This is called the 'screening' assay. If the optical density (OD) in the EIA exceeds a preset cut-off, the donor animal is deemed a 'suspect' reactor.

All suspect reactors in the screening assay are then subjected to a 'full series' assay. Plasma from the blood samples incubated with PBS, avian PPD and bovine PPD are all simultaneously assayed for IFN- γ and the responses compared. A commercial EIA kit for the determination of IFN- γ is available from CSL Ltd.

4.1. Collection of Blood Samples

All cattle must be individually identifiable either by ear-tag, tail-tag or tattoo.

Blood is collected into 10 mL, sterile, evacuated tubes [Vacutainer, Becton Dickinson Cat. No. 6848; 80 Rushdale Road, Knoxfield, Vic. 3180; Tel. (03) 764 2444; Fax (03) 764 2550] containing heparin as anticoagulant (EDTA or citrate MUST NOT be used). Blood tubes MUST be filled to at least half way (5 mL) as 4.5 mL of blood is required for the assay. All blood tubes should be labelled sequentially (e.g. 1-120) and these numbers related to animal identification on an identification sheet to accompany the submission form. The time of collection of the first sample should be recorded. A new sterile 18 g Vacutainer needle (1 inch, 25 mm; Becton Dickinson Cat. No. 5747) should be used for each animal. Tail bleeding is recommended to facilitate bleeding of large numbers of animals but for small groups jugular venipuncture may be the method of choice. There is no difference in reactivity between venous and arterial blood in the IFN- γ assay. Blood must not be collected post mortem as this has been shown to reduce the sensitivity of the assay (Rothel *et al.*, 1992).

4.2. Transport and Storage of Blood Samples

All blood samples must be transported to the testing laboratory within 12 hours of collection (within eight hours is preferable). There is a marked loss of reactivity in blood stored for longer periods (Rothel *et al.*, 1992). Blood samples should be stored and transported at 20-25°C and under no circumstances should

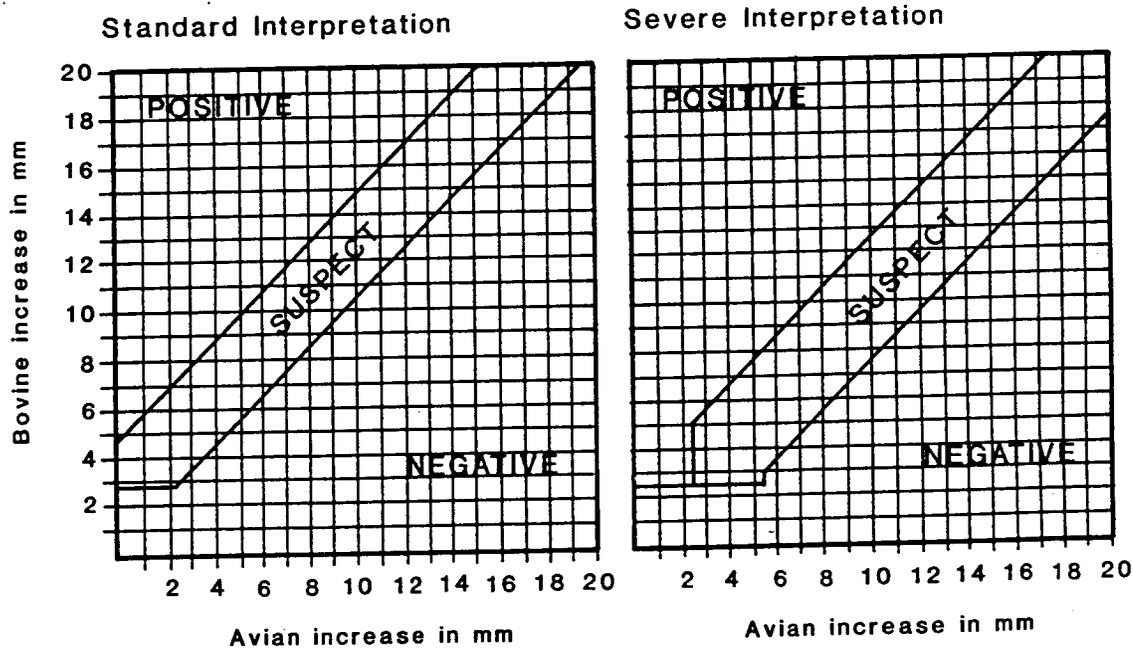


Figure 1. Interpretation of the comparative intradermal tuberculin test.

they be refrigerated. If the ambient temperature is greater than 30°C, blood samples should be stored in an insulated container suitably cooled with a freezer block.

4.3. Incubation of Whole Blood Cultures with Tuberculin

The equipment required for the setting up of the whole blood cultures is listed in 7.1. Blood samples should be mixed well (by inverting tubes several times) before dispensing. Aliquots (1.5 mL) of each blood sample are dispensed aseptically into each of three wells of a sterile 24-well tissue culture tray. To minimise the possibility of contamination of the blood with air-borne bacteria, blood should be dispensed, using sterile pipettes, in a biohazard cabinet. If a sterile cabinet is not available, blood may be dispensed in a clean room with strict adherence to aseptic practice.

Sterile, dialysed bovine and avian PPDs (CSL, 300 µg/mL) are supplied with the IFN-γ EIA kits. PBS (0.01 mol/L, pH 7.3) is also required and should be prepared and sterilised before use. To each aliquot of blood, 100 µL of either PBS (nil antigen control), bovine PPD or avian PPD is added using sterile pipettor tips or sterile multiple dose dispensers such as the Eppendorf Combitip fitted with sterile 5 mL tips. Plates are covered with lids and then incubated at 37°C for a minimum of 16 hours in a humidified atmosphere of air alone or air containing 5% carbon dioxide (CO₂).

4.4. Harvesting of Plasma after Incubation

The laboratory equipment and plasticware necessary for the harvesting of plasma samples are listed in 7.2.

After incubation, culture trays are centrifuged at 1000 g for 10–15 min, using a suitable rotor which can hold 24-well trays. Plasma is removed from above the sedimented red cells using an Oxford type pipettor (100–1000 µL).

Approximately 150 µL of plasma harvested from

the blood stimulated with bovine PPD (bovine PPD sample) is transferred into a 96-well tissue culture or ELISA plate. The format shown in Fig. 2 is recommended for storage of these bovine PPD samples as this enables the use of 12-channel pipettors to transfer samples to the appropriate wells of IFN-γ EIA plates. The culture trays, containing the remaining unharvested plasma from blood incubated with bovine PPD and that incubated with PBS and avian PPD (PBS and avian PPD samples) should be temporarily stored at room temperature until the results of the screening assay are obtained and analysed.

The contamination of the plasma with small amounts of erythrocytes during harvesting has no effect on the IFN-γ EIA. Similarly, slight haemolysis of blood samples has little effect on the IFN-γ EIA.

As soon as the plasmas from the bovine PPD samples are harvested, they are assayed in duplicate for the presence of IFN-γ using the IFN-γ EIA as described in 4.5.

The results from this 'screening' assay are then analysed as described in 4.6.1. For all animals that are positive on initial screening it is necessary to then harvest plasma from the PBS, avian PPD and bovine PPD wells. These plasma samples should be harvested AS SOON AS POSSIBLE after completion of the screening assay. Plasma (>200 µL) is removed from above the sedimented red cells using an Oxford type pipettor (100–1000 µL). One pipettor tip can be used for the PBS and avian PPD samples (in that order) with a fresh tip used for the bovine PPD sample. The plasma samples should be transferred into 96-well (8 x 12 format) storage racks. The suggested format for storage of these samples is shown in Fig. 3. This again enables the use of 12-channel pipettors to transfer samples to the appropriate wells of IFN-γ EIA plates.

The plasma samples are then assayed for IFN-γ in the EIA ('Full assay', see 4.6.) and the results analysed as in 4.6.2.

		COLUMN											
		1	2	3	4	5	6	7	8	9	10	11	12
ROW	A	1	2	3	4	5	6	7	8	9	10	11	12
	B	13	14	15	16	17	18	19	20	21	22	23	24
	C	25	26	27	XX	XX	XX	28	29	30	31	32	33
	D	34	35	36	37	38	39	40	41	42	43	44	45
	E	46	47	48	49	50	51	52	53	54	55	56	57
	F	58	59	60	61	62	63	64	65	66	67	68	69
	G	70	71	72	XX	XX	XX	73	74	75	76	77	78
	H	79	80	81	82	83	84	85	86	87	88	89	90

Figure 2. Pattern for the storage of bovine PPD samples prior to 'screening' assay in the IFN-γ EIA. Bovine PPD samples from 90 cattle can be harvested into 1 x 96-well tray. Wells C4, C5, C6, G4, G5 and G6 (indicated by XX) are left empty and are used for positive and negative controls in the EIA plate.

		COLUMN											
		1	2	3	4	5	6	7	8	9	10	11	12
ROW	A	1N	1A	1B	2N	2A	2B	3N	3A	3B	4N	4A	4B
	B	5N	5A	5B	6N	6A	6B	7N	7A	7B	8N	8A	8B
	C	9N	9A	9B	XX	XX	XX	10N	10A	10B	11N	11A	11B
	D	12N	12A	12B	13N	13A	13B	14N	14A	14B	15N	15A	15B
	E	16N	16A	16B	17N	17A	17B	18N	18A	18B	19N	19A	19B
	F	20N	20A	20B	21N	21A	21B	22N	22A	22B	23N	23A	23B
	G	24N	24A	24B	XX	XX	XX	25N	25A	25B	26N	26A	26B
	H	27N	27A	27B	28N	28A	28B	29N	29A	29B	30N	30A	30B

Figure 3. Storage pattern for PBS, avian PPD and bovine PPD samples. The plasma samples from 30 animals can be harvested into each 96-well storage rack. Wells C4, C5, C6, G4, G5 and G6 (indicated by XX) are left empty, but are used for positive and negative controls in the EIA plates.

4.5. Interferon- γ Enzyme Immunoassay

The EIA for bovine IFN- γ is manufactured by CSL Ltd and should be performed strictly to the manufacturer's instructions. Laboratory equipment required to perform the EIA is listed in 7.3.

The EIA involves three separate stages.

4.5.1. Stage 1

The IFN- γ present in plasma samples reacts with antibodies to bovine IFN- γ bound to a solid support. Unbound material is removed by washing after incubation for one hour.

4.5.2. Stage 2

Conjugate (horseradish peroxidase labelled anti-bovine IFN- γ) reacts with IFN- γ bound to the antibody coated to a solid support. Unreacted conjugate is removed by washing after incubation for one hour.

4.5.3. Stage 3

Enzyme substrate (for horseradish peroxidase) is added. The rate of conversion of substrate is proportional to the amount of bound IFN- γ . The reaction is terminated after 30 min and the amount of colour development estimated spectrophotometrically.

All test samples should be assayed in duplicate, this allows 45 test plasmas to be assayed per plate. The suggested plasma storage patterns for both the 'screening' and 'full series' assay are shown in Figs 2 and 3, respectively. These patterns facilitate the transfer of samples to the EIA plates using a 12-channel pipettor. Plasma samples (50 μ L) from row A in the 96-well storage rack (or tray) are transferred to row A and row B in the EIA plate using a 12-channel pipettor. Similarly, plasma samples in rows B, C and D of the storage rack are transferred to rows C and D, E and F, and G and H of the EIA plate, respectively. Plasma samples from rows E, F, G and H of the storage rack are transferred, using the same method, to a second IFN- γ EIA plate. Therefore, for each storage rack of samples, two IFN- γ EIA plates are required.

After transfer of test plasma samples to the IFN- γ EIA plates, wells E4-E6 and F4-F6 of the EIA plates will be empty. These wells are used to assay the positive and negative controls in triplicate (row E for positive plasma and row F for negative plasma). The positive and negative controls are supplied with the EIA plates. There are some areas of the EIA procedure, although addressed in the manufacturers instructions, that require special attention. These are listed below.

- (a) Thorough washing of IFN- γ EIA plates is critical to the performance of the assay. At least six washes should be performed at each step, thoroughly 'flicking out' wash buffer from the plates between each wash. Automatic washers or washers that aspirate

the buffer from wells should not be used as they commonly result in erroneous results when used for sensitive assay systems. This is probably due to unsatisfactory removal of wash buffer from wells between washes.

- (b) Take care to ensure that all EIA plates and reagents are brought to room temperature before beginning the EIA. This ensures optimal performance.
- (c) Polystyrene containers or pipettes MUST NOT be used for the preparation or dilution of any EIA reagent as the polystyrene reacts with some reagents in the EIA producing erroneous results. Polypropylene containers are recommended.
- (d) It is important that high quality deionised or distilled water is used to reconstitute and dilute reagents and wash buffer, as horseradish peroxidase is readily inactivated by pollutants common in laboratory water supplies.

4.6. Interpretation of Results

As the IFN- γ assay system gives a quantitative result it is possible to adjust the sensitivity of the assay to detect tuberculous cattle, by altering the 'cut-off point' used to assess the EIA data.

However, by increasing the sensitivity of any test a commensurate decrease in specificity is usually observed and *vice versa*. The use of various criteria for calculating sensitivity and specificity values for the IFN- γ assay, as obtained during field trialing of the assay in 1989-90, has been reported by Wood *et al.* (1991). The IFN- γ EIA interpretation method as outlined below should be used to attain maximum sensitivity. In the final stages of eradicating tuberculosis in Australian cattle, it is presumed the highest possible sensitivity for the assay will be required.

4.6.1. 'Screening' Assay

An animal is deemed to be positive in the 'screening' assay if the mean OD in the EIA of the bovine PPD sample is greater than or equal to the mean OD for the plate negative control + 0.05. i.e.

$$\text{BOVINE PPD SAMPLE OD} \\ \geq \text{PLATE NEGATIVE CONTROL OD} + 0.05$$

4.6.2. 'Full Series' Assay

If an animal is positive in the screening assay, the PBS, avian PPD and bovine PPD samples are then assayed in the full series assay to determine the specificity of the response. An animal is a POSITIVE reactor, in the full series assay, if the mean OD of its bovine PPD sample is greater than or equal to the mean OD for the plate negative control + 0.05 (as for the screening assay) AND the mean OD of the bovine PPD sample is greater than the mean OD for both the PBS and avian PPD samples.

Computer programs have been written to analyse the data from the screening and full series assays. These programs are available on request from: Jim Rothel, CSIRO Division of Animal Health, Private Bag No.1, Parkville, Vic. 3052; Tel. (03) 342 9700; Fax (03) 347 4042.

5. Repeat Testing

Intradermal tuberculin testing in cattle alters the immunoreactivity of animals to repeat testing for up to 60 days (Radunz and Lepper, 1985). Tuberculin testing has also been shown to affect the immunoreactivity of cattle infected with *M. bovis* towards the IFN- γ assay for up to 60 days (Rothel *et al.*, 1992). Therefore, cattle must not be subjected to either the tuberculin test or the IFN- γ assay for at least 60 days after a tuberculin test.

As the IFN- γ assay does not involve the injection of tuberculin into the animal, it has no effect on the immunoreactivity of cattle. Therefore, cattle can be retested with either the IFN- γ assay or the tuberculin test at any time after a IFN- γ assay provided they have not been tuberculin tested within the previous 60 days.

6. References

- Radunz, B.L., and Lepper, A.W.D. (1985). Suppression of skin reactivity to bovine tuberculin in repeat tests. *Australian Veterinary Journal* 62, 191-4.
- Rothel, J.S., Jones, S.L., Corner, L.A., Cox, J.C., and Wood, P.R. (1990). A sandwich enzyme immunoassay for bovine interferon- γ and its use for the detection of tuberculosis in cattle. *Australian Veterinary Journal* 67, 134-7.
- Rothel, J.S., Jones, S.L., Corner, L.A., Cox, J.C., and Wood, P.R. (1992). The interferon- γ assay for diagnosis of bovine tuberculosis in cattle: conditions affecting the production of interferon- γ in whole blood culture. *Australian Veterinary Journal* 69, 1-4.
- Wood, P.R., Corner, L.A., Rothel, J.S., Baldock, C., Jones, S.L., Cousins, D.B., McCormick, B.S., Francis, B.R., Creeper, J., and Tweddle, N.E. (1991). Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. *Australian Veterinary Journal* 68, 286-90.

7. Appendixes

7.1. Appendix 1 — Equipment Required for the Interferon- γ Whole Blood Culture

- Disposable 5 or 10 mL pipettes (sterile)
- 24-Well tissue culture trays (sterile): suggest Nunclon (Nunc Cat. No. 143982)
- Automatic pipette filler (to dispense blood)
- Pipetter to dispense antigens (100 μ L/well): suggest Eppendorf Multipet (Eppendorf Cat. No. 4780000.010)
- Combitip (Eppendorf Cat. No. 0030048.237) tips for Multipet dispenser (5 mL)
- Sterile PBS (0.01 mol/L, pH 7.2)
- Laminar flow or Biohazard cabinet (not essential).
- 37°C, humidified incubator (CO₂ not essential).

7.2. Appendix 2 — Equipment Required for Harvesting Plasma Samples

- Oxford type pipettor (0.1-1.0 mL).
- Tips to fit 0.1-1.0 mL pipettor.
- 96-Well microtitre trays for storage of plasma samples from 'screening' assay
- 96-Well format racks (suggest Bio-Rad, 960 Titertube, Cat. No. 223-9390) for storage of plasma samples from 'full series' assay
- Centrifuge to spin 24-well trays.

7.3. Appendix 3 — Equipment Required for the Interferon- γ Enzyme Immunoassay

- Accurate, replaceable-tip variable-volume pipettes (to deliver from 50 to 1000 μ L)
- Graduated 1, 5 and 10 mL pipettes
- Measuring cylinders — 100 mL, 1 L and 2 L
- Suitable manually operated microtitre plate washer, e.g. Nunc-Immuno Wash 8/12 (Nunc Cat. No. 470173/455492)
- Multichannel pipettor (to deliver 50 μ L and 100 μ L)
- Suitable microtitre plate reader. This reader MUST be fitted with a 450 nm filter.

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Bovine Tuberculosis

Pathology and Bacteriology

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1. Introduction

In the early stages of tuberculosis control great reliance was placed on detecting macroscopic lesions during gross post mortem examination. If there was some doubt as to the cause of the lesion, histopathological examination was employed. Cultural examinations were rarely done. When the national bovine tuberculosis eradication campaign commenced, the need for a definitive diagnosis became more frequent and routine culturing of all suspected lesions became commonplace. More recently the importance of non-visible lesion (NVL) reactors and single reactors in herds thought to be free from tuberculosis (singleton reactors) has been recognised and exhaustive cultural examination of tissues from these animals has been used to clarify the status of the herd.

A tentative diagnosis of bovine tuberculosis can be made following the finding of typical lesions during necropsy. These lesions should be collected for laboratory confirmation of the diagnosis. A definitive diagnosis can only be made by isolating *Mycobacterium bovis*.

Under Australian conditions, most tuberculosis infections are acquired by inhalation. Between 70% and 90% of lesions are found in either the lymph nodes of the head or in the thoracic cavity. Lesions are most often found in organs of the reticuloendothelial system including the lung, spleen and liver, but may occur at virtually any site in the body, so all organ systems need to be examined.

On a property where bovine tuberculosis is known to exist and where a change of status is not dependent on the outcome of the necropsy, the examination may be limited to the lungs and the lymph nodes of the head, thorax, and mesentery. However, if a change of status is possible a thorough examination is required.

The procedure is modified where it has been established that non-specific reactors are known to be a problem and the cause is to be investigated, or a singleton reactor is detected. Specimens are collected using a strict aseptic technique that excludes slicing at the time of collection. The examination of these tissues for the presence of lesions is then done in a laboratory or in clean surroundings to prevent contamination. If no lesions are found the tissues should be cultured to determine if *M. bovis* infection is present. If lesions are found, up to three are taken for culture.

2. Pathology

The detection of macroscopic lesions at necropsy is an important aspect of the diagnosis of bovine tuberculosis. Frequently a presumptive diagnosis of bovine tuberculosis is made on the basis of gross pathology and examination of smears or histological sections made from lesions.

2.1. Gross Pathology

Lesions may vary in size from 1 mm to greater than 10 cm diameter. There may be single lesions in lymph nodes or a primary complex, i.e. lesions in a parenchymatous organ and a lymph node draining the organ.

Lesions, also called tubercles, have the following characteristics. Most appear as firm or hard, white, grey or yellow nodules. The cut surface usually shows a yellowish, caseous centre which is dry and firm. Calcification is common, particularly in lymph nodes, and in sectioning the lesion a gritty sensation and grating sound indicates its occurrence. Conglomerate tubercles, formed by the growth and coalescence of one or more adjacent tubercles may occur over the pleural or peritoneal surfaces. Metastases give rise to myriad tubercles all of the same size, usually 2–3 mm diameter. Old lesions may be encapsulated by connective tissue, heavily calcified and inspissated.

2.2. Smears from Lesions

Smears are prepared from the caseous material lining the inner wall of the lesion and stained for acid-fast bacteria. Detection of acid-fast bacteria resembling *M. bovis* may give an early presumptive diagnosis of bovine tuberculosis. A recommended Ziehl-Neelsen method (carbol fuchsin) (see 8.2.1.) or comparable fluorochrome (auramine-O) procedure (see 8.2.2.) are used to stain the smears. In smears of lesions stained by the Ziehl-Neelsen method *M. bovis* appear as red, medium length acid-fast rods, singly or in clumps. With the auramine-O stain acid-fast bacteria appear as bright yellow rods against a dark background.

2.3. Histopathology

Sections for histological examination are prepared using routine procedures and sections are stained for normal tissue elements, e.g. using haematoxylin and eosin or azure-eosin, and also for acid-fast bacteria using a recommended Ziehl-Neelsen method (carbol fuchsin) or comparable fluorochrome (auramine-O) (see 8.2.3.) procedure. Some lesions may require decalcification prior to processing. Where time permits or good histological detail is required a saturated solution of ethylenediamine tetraacetic acid (disodium salt) (EDTA) is used to decalcify the tissue. If prompt histological diagnosis is required decalcification can be achieved in 24 hours with sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) -formic acid (CH_2O_2) solution but at the expense of some histological detail (Luna, 1968). Decalcification with nitric acid (HNO_3) should be avoided.

M. bovis infection evokes a characteristic granulomatous reaction, the tubercle, with the following characteristics.

- (a) A central area of necrosis.

- (b) Some degree of calcification that usually occurs in the centre of the caseated area.
- (c) The caseated area is surrounded by a zone of epithelioid cells.
- (d) Usually Langhan's giant cells are found on the margin of the zone of epithelioid cells, and elsewhere.
- (e) A zone of lymphocytes, macrophages and plasma cells is found towards the periphery of the tubercle.
- (f) The tubercle is generally encapsulated by fibrous tissue.
- (g) *M. bovis* appear in Ziehl-Neelsen stained sections as red medium-length rods, singly or in clumps. They are found in the cytoplasm of the macrophages and giant cells on the periphery of the lesion and scattered through the necrotic debris in the centre of the lesion. They are generally only present in low numbers in most natural cases of bovine tuberculosis.

2.4. Differential Diagnosis

Several conditions may be confused, both macroscopically and microscopically, with tuberculosis. The most important of these and the histological characteristics allowing their differentiation are given below.

2.4.1. *Rhodococcus equi* Granuloma

- (a) Neutrophil infiltration of the necrotic zone is usually heavy and uniform but, in some cases, only small foci of neutrophils are seen at the periphery of the necrotic area.
- (b) Calcification of the caseated area is common, but more likely to be located at the edge than centrally.
- (c) In lesions with little neutrophil infiltration, confident histological differentiation from tuberculosis is not possible. In these cases a Gram stain must be used to demonstrate the Gram-positive coccobacilli.

2.4.2. *Nocardia-Streptomyces* and Other Fungal Granulomas

- (a) Giant cells are often very numerous, but tend to be of the foreign-body type. This is usually the most important distinguishing feature.
- (b) Calcification is less common than in tuberculosis.
- (c) Neutrophils are often associated with the necrotic area.
- (d) The causative organism is clearly visible in Gram or Gomori methenamine silver (GMS) (Grocott, 1955) stained sections.

2.4.3. Oil Granulomas

- (a) Mineralisation does not occur.
- (b) Oil droplets are usually visible.

2.4.4. Hydatid Cysts

Occasionally, degenerating hydatid cysts may take on many of the histological features of the tubercle including caseation, necrosis, calcification and the formation of Langhan's and/or foreign-body giant cells. Differential diagnosis depends on identification of the outer non-nucleated layer of the cyst wall. This layer is composed of many delicate laminations which are usually still visible even in very old lesions in which only fragments of the cyst wall remain. Eosinophils may be numerous.

2.4.5. Other Conditions

Neoplasms, club-forming granulomas and abscesses may cause lesions that are macroscopically similar to tuberculosis, but most are readily distinguishable histologically.

3. Collection of Specimens for Laboratory Diagnosis

3.1. Necropsy Technique

The necropsy examination for diagnosing bovine tuberculosis must be thorough and all tissues listed in Table 1 must be identified and examined. A reactor cannot legitimately be called a false-positive or a non-visible lesion (NVL) reactor unless all those tissues have been thoroughly examined. This is particularly important where the results of the necropsy on a reactor animal has the potential to change the status of the herd.

Table 1. Tissues to be examined for lesions in cattle reacting to a tuberculin test
In=lymph node

Head:	
Mandibular In	Left and right
Parotid In	Left and right
Medial retropharyngeal In	Left and right
Lateral retropharyngeal (atlantal) In	Left and right
Tonsils	
Thorax:	
Mediastinal In	Anterior and posterior
Tracheobronchial (bronchial) In	Left, right, cranial and medial
Lungs	
Abdomen:	
Liver	
Hepatic In	
Spleen.	
mesenteric In along the entire length of the gastrointestinal tract	
Kidneys	
Carcass:	
Caudal cervical (prescapular) In	Left and right
Subiliac (prefemoral) In	Left and right
Internal iliac In	Left and right
Medial iliac In	Left and right
Lateral iliac (deep inguinal) In	Left and right
Gluteal (ischiatric) In	Left and right
Sacral In	Left and right
Superficial inguinal (supramammary or scrotal) In	Left and right
Udder or scrotal contents and seminal vesicles	

Protective clothing and gloves should be worn by the operator during the examination. The dissection of the carcass should be done with due care, such that if a lesion is encountered the operator is not put at risk of becoming infected. Lymph nodes are examined by slicing at 2 mm intervals and the cut surfaces examined carefully for lesions. The surface of the lungs are examined for lesions and then palpated and sliced at 20–50 mm intervals. The surface of the spleen and liver are examined for lesions and sliced at 20–50 mm intervals. The knife used to open and dissect the carcass should not be used to slice tissues for detection of lesions without it first being sterilised.

3.2. Collection and Submission of Specimens

Sections of lesions are submitted for mycobacteriological and histopathological examination. Up to three lesions from each animal are submitted to ensure a correct diagnosis is made. Lesions should be divided into two equally representative portions and submitted, one refrigerated in a sterile leak-proof container and the other in 10% buffered formalin (CH₂O, Lillie and Fullmer, 1976) If a lesion is too small to divide, collection for bacteriology is given preference over that for histopathology.

3.2.1. Non-visible Lesion Reactors and Singleton Reactors

M. bovis may be isolated from a small proportion of non-visible lesion (NVL) reactors. As *M. bovis* is present in low numbers in infected tissues in which no lesions have developed, it is important to minimise contamination that may reduce the sensitivity of culture techniques. Aseptic collection of a large number of specimens (Table 2) is therefore desirable if a meaningful mycobacterial culture result is to be achieved. Where a single reactor is detected in a test of a herd that has no history of bovine tuberculosis or where a change in status of a herd may rest on the results of the examination, an extensive bacteriological examination of the reactor is warranted. In this instance too, aseptic collection of a wide range of specimens is necessary.

The aseptic collection procedure is designed to minimise contamination of specimens with environmental fungi and bacteria, including other mycobacteria. Slicing of lymph nodes in the field increases the risk that specimens will become contaminated which in turn may lead to false conclusions regarding the presence of *M. bovis* or other mycobacteria.

The necropsy is performed with knives that have been cleaned and disinfected by boiling for five minutes or with chemical disinfectants. Knives are disinfected frequently during dissection of the carcass, particularly when approaching tissues nominated for collection. Specimens for bacteriological examination are collected

using sterile instruments, scissors and forceps sterilised by boiling or instruments that are supplied in sterile packs, e.g. scalpel blades. Tissues with gross surface contamination may be flamed before collection of the sample.

Lymph nodes are dissected free of surrounding tissue and fat *but the capsule of the node should not be cut*. They should then be forwarded to the laboratory unsliced. Portions of liver, lungs and spleen containing suspect lesions are also collected aseptically.

If it is necessary to know immediately if small lesions are present in the nodes that have been collected then they may be examined in the field. The procedure used varies between States. Some States advocate palpation and others the slicing of the nodes in a clean environment. Both of these procedures have the potential to introduce contaminants into the specimen, with palpation the lesser of the two evils. Palpation should be done with gloved hands, disinfected between specimens. Nodes may be sliced in a draught-free, dust-free environment. The specimens are sliced at 2 mm intervals with sterile instruments, on an impervious surface sterilised with 70% ethanol or methylated spirits before commencing and between each sample.

3.2.2. Specimens for Bacteriological Diagnosis

Sections of lesions are collected using an aseptic technique. The sections are placed in sterile leak-proof containers and refrigerated. If the specimen will not reach the laboratory within 24 hours of collection, the specimen is placed in either sterile saturated borate solution or coated with sodium tetraborate (borax, Na₂B₄O₇) powder and refrigerated (about 4°C). Specimens not cultured within four days of collection should be frozen (-10°C or colder). These must remain frozen until cultured in a laboratory. It is important that the specimens remain frozen during any transport.

Table 2. The following lymph nodes are collected for culture from singleton reactors and from reactors in a herd with a history of non-visible lesion reactors
In = lymph node

Priority	Tissue	
Essential	Medial retropharyngeal In	Left and right
	Tracheobronchial (bronchial) In	Left and right
	Mediastinal In	Anterior and posterior
Highly desirable	Tracheobronchial (bronchial) In	Cranial and medial
Desirable	Other thoracic lymph nodes	
	Mandibular In	Left and right
	Parotid In	Left and right
	Lateral retropharyngeal In	Left and right
	Medial iliac In	Left and right
	Mesenteric In from the region of the duodenum, jejunum and ileum	
Superficial inguinal (mammary or scrotal) In	Left and right	

3.2.3. Specimens for Histopathological Diagnosis

Thick (5 mm) sections of lesions are placed in 10% buffered formalin. The section should include both normal and lesioned tissue and should not be more than 2 cm². The volume of formalin should be 10 times the volume of the specimen.

4. Laboratory Procedures for the Isolation of *Mycobacterium bovis*

M. bovis may be present in tissue in very small numbers or the bacteria in small lesions (such as those present in tissues of NVL reactors) may be diluted by the surrounding tissue when processed. The chances of isolating *M. bovis* are improved by increasing the amount of material cultured. This is most easily achieved by increasing the number of slopes used. Each specimen should be cultured separately onto four slopes of media. It is recommended that at least two different media bases be used, i.e. one egg based and one agar based.

The isolation of *M. bovis* from nasal swabs of infected animals has been reported (Kantor and Roswurm, 1978; McIlroy *et al.*, 1986).

Investigations in Australia with nasal swabs taken from experimentally infected cattle with advanced pulmonary disease (L.A. Corner, unpublished data 1987) failed to demonstrate any value in this procedure.

For the isolation of *M. bovis*, specimens are submitted fresh, preferably chilled. Frozen specimens should only be submitted if delays in transport are expected. Frozen specimens are thawed at 4°C overnight or at room temperature on the day of processing. Borate powder is washed off the specimen with sterile buffer, saline or distilled water. The specimen is macerated before being cultured. If there is a high risk that the specimen may be contaminated with other microorganisms, the material can be decontaminated prior to inoculation of the culture media.

Handling and manipulation of infectious material, both tissue specimens and cultures, should be done in a Class I or Class II biohazard cabinet to protect both the specimen from contamination and the operator from infection.

4.1. Description

M. bovis are acid-fast bacteria and although not readily stained by Grams method, are considered Gram positive. On smears made from colonies they are coccobacilli or short rods, 0.3–0.6 µm by 1–4 µm. Cording may be observed, especially in liquid cultures. They are microaerophilic, slow growing bacteria requiring a minimum of 14 days for colonies to become visible. On egg media the typical colony is small, rounded, pale yellow to buff with irregular edges and a granular surface. On agar medium they are white, thin, rough and flat with a central mound.

4.2. Culture Media

Field strains of *M. bovis* require media enriched with serum or egg yolk or egg albumin, for growth on primary isolation. The media used are either egg based or agar based. For primary isolation it is recommended that two slopes each of an egg-based medium and an agar-based medium be used. On egg-medium colonies the strains take an average of 35 days to become visible but on agar medium growth they become visible on an average of four to six days earlier. Media for primary isolation usually contain dyes to inhibit the growth of contaminants. For long term maintenance of cultures, egg-based media are preferred. Two types of egg medium have been found to give satisfactory results, Lowenstein-Jensen medium with pyruvate, but without glycerol and Stonebrink's medium (see 8.1.). There are two agar media that give similar results, modified Middlebrook 7H11 (Gallagher and Horwill, 1977) (see 8.1.) and tuberculosis blood agar (also called B83 agar) (Cousins *et al.*, 1989) (see 8.1.). *M. bovis* grows more rapidly on the modified Middlebrook 7H11 medium but on egg media more colonies grow and the medium is less susceptible to the growth of contaminants.

The egg-based media and modified Middlebrook 7H11 medium are made selective by adding dyes to the base media, most commonly malachite green and crystal violet. Selective media containing antibiotics have been developed by Mitchison *et al.* (1973) and P. Anargyros (pers. comm., 1987) (see 8.1.). There is some inhibition of *M. bovis* by these antibiotic mixtures but they may be useful with some contaminated specimens.

The media recommended here are for the isolation and maintenance of *M. bovis*. If other mycobacteria are being cultivated additional media may be required. If liquid media is used, Kirchner broth or Selective Kirchner broth (see 8.1.) are suitable. Pyruvate or glucose should be substituted for glycerol in the recipe.

4.3. Maceration

Prior to culture the specimen is homogenised or macerated. From 5 to 10 g of lesion material may be processed depending on the method of maceration. If the mass of the lesion exceeds 10 g, then part of the fibrous capsule of the lesion and some of the caseous material is cultured. Maceration can be done in a Colworth Stomacher (well suited to lymph nodes) or a tissue blender. For tissues without visible lesions, the superficial fat is trimmed from the surface of the specimen using sterile instruments, and the entire lymph node, or up to 10 g, is sliced into small pieces. The pieces (0.5–1.0 cm³) are macerated in 2–10 volumes of sterile water or saline. The macerated tissue, excluding large tissue pieces, is then decontaminated.

4.4. Decontamination Procedures

Decontamination of specimens is often necessary to permit the isolation of *M. bovis* from some specimens. Specimens collected aseptically should be cultured both before and after decontamination. The decontamination reagents chosen should have minimal effect on the viability of *M. bovis* in the specimen while at the same time rendering contaminating organisms non-viable. Hexadecylpyridinium chloride (HPC) at a final concentration of 0.075% is recommended for this purpose. HPC is added to the macerated tissue and then incubated at room temperature for 30 min. Maximum total contact time of 60 min including centrifugation time, should not be exceeded because losses of *M. bovis* become unacceptably high. Decontamination with reagents such as HPC at 0.75% or sodium hydroxide (NaOH) (2%) and oxalic acid (C₂H₂O₄) (5%) are not recommended for routine decontamination procedures because of their toxicity to *M. bovis*. These latter reagents may be employed with individual specimens where HPC 0.075% has failed to control contamination. HPC at 0.75% is preferred to sodium hydroxide and oxalic acid.

When sodium hydroxide is used, the macerated tissue is exposed to the reagent for 30 min and then is neutralised with acid, e.g. 10% hydrochloric acid (HCl), 10% sulfuric acid, H₂SO₄ or 10% phosphoric acid (H₃PO₄) using phenol red as the indicator. Tissue decontaminated with oxalic acid is cultured with or without neutralisation.

4.5. Centrifugation

Concentration of the macerated tissue is achieved by centrifugation at 1800–3000 g for 10–20 min. Centrifugation at a higher speed for a shorter time is preferred. The supernate is discarded and the sediment inoculated onto the media with a cotton wool applicator stick or the sediment can be resuspended in a small volume of the supernate and the media inoculated with 0.1–0.2 mL.

4.6. Incubation

The inoculated media is incubated at 37°C in air, with or without the addition of carbon dioxide (CO₂) (maximum 5%). The media are examined daily for the first week (if practicable) and then weekly. Cultures should be maintained, optimally, for 12 weeks. Incubation for eight weeks is an acceptable minimum period.

4.7. Laboratory Animal Inoculation

Laboratory animal inoculation for the primary isolation of *M. bovis* is not recommended. For contaminated specimens, where this procedure has been most widely employed in the past, the culture methods outlined above are at least as sensitive as animal inoculation.

4.8. Identification

Isolates of *M. bovis* should have the following characteristics.

4.8.1. Colony characteristics

On egg media after 21 days or more incubation the colonies are small, rounded, white to pale yellow or buff with irregular edges and a granular surface. On agar media after 14 days the colonies are thin, flat with a central mound, granular and white.

4.8.2. Growth Temperature and Speed

Grow at 37°C. Grow slowly and on subculture require at least 14 days for individual colonies to appear on egg media

4.8.3. Cell Morphology

Acid-fast coccobacilli or short rods; cording is frequently observed

4.8.4. Biochemical Tests

Niacin negative. Inhibited by: 2-thiophene carboxylic hydrazide; para-aminosalicylic acid; neotetrazolium; glycerol (most strains).

4.8.5. Immunoperoxidase

Positive binding of monoclonal antibody 4C3/17 [antibody is available from Agen Biomedical Ltd, PO Box 391, Acacia Ridge, Qld 4110, Australia. Tel. (07) 273 6266; Fax (07) 273 6224].

5. Safety

Apart from the usual hazards of handling infectious material that exist in all microbiology laboratories, there are potentially more serious risks in handling material infected with pathogenic mycobacteria. Current standards for the design of microbiology laboratories and the procedures for handling infectious material, reduce the risk of acquiring infection while working in these environments.

In field work the risk of infection arises mainly from exposure to infectious material from lesions. These risks can be minimised by taking suitable precautions.

A safety program for personnel at risk of exposure to *M. bovis*, including both field and laboratory staff, should cover:

- an ongoing health monitoring procedure for all staff at risk;
- adequate instruction of personnel as to the sources of danger;
- use of disinfectants known to be mycobacteriocidal;
- supervision of inexperienced personnel, and for laboratory staff in particular;
- availability of effective containment equipment;
- appropriate arrangements for sterilising infectious waste.

5.1. Monitoring the Health of Personnel at Risk
Personnel who are likely to be exposed to *M. bovis*, should be in good health and show evidence of 'immunity' to tuberculosis by being positive on skin test. Those who are negative should be vaccinated with BCG. Annual chest X-rays should be carried out.

5.2. Sources of Infection

For workers conducting autopsies in the field the major source of risk is lesion material. The risks to the person conducting the examination are associated with accidental contamination of wounds, inhalation of infectious droplets generated when cutting into a lesion or ingestion resulting from poor personal hygiene.

For laboratory workers the risks are similar to those mentioned above but the major risk is from aerosols. The generation of infectious aerosols is associated with opening specimen containers, pouring of liquids or macerated specimens, centrifugation, use of pipettes, preparing smears and flaming of loops.

5.3. Disinfectants

Many disinfectants, even those nominated as 'hospital' disinfectants, are not effective against mycobacteria. Phenolic and synthetic phenolic disinfectants are recommended, e.g. Medol at 2%.

A procedure for testing disinfectants against *M. tuberculosis* has been developed at the State Health Laboratory, Brisbane.

For further information contact:

Mr D. Dawson, Tuberculosis Section, State Health Laboratory, PO Box 495, Brisbane, Qld 4001. Tel. (07) 224 5528; Fax (07) 2219737.

Medol is available from: Australian Laboratory Services P/L, Box 193, PO Rockdale, NSW 2216. Tel. (02) 567 5258, (008) 252 286; Fax (02) 597 2232.

5.4. Supervision of Personnel

Inexperienced workers should be instructed in all aspects of safe working practices and given specific instructions regarding the particular risks associated with tuberculosis. They should be supervised closely during their initial period of work until they are competent.

5.5. Containment Facilities

Culturing of material for the isolation of *M. bovis* and the handling of *M. bovis* cultures should be done in a containment laboratory. Such a laboratory is not open to public areas of the general laboratory and should have an independent air exhaust system. Conditioned air may be ducted into the containment area but the air should not be recirculated. For effective containment, all operations within the laboratory that could result in the production of aerosols must be done in a Biological Containment Cabinet. The cabinets should be located away from busy

work areas in the laboratory. A Class I cabinet, that protects the worker rather than the material, or a Class II cabinet, that protects both the worker and the material, are suitable. These cabinets require regular (annual) inspections and performance testing.

Centrifuges used in culturing should be housed in a biological safety cabinet or have sealed rotors or buckets. After spinning, the rotor or bucket is opened only in a safety cabinet.

5.6. Sterilisation of Infectious Material

Infectious material, such as lesion material, contaminated laboratoryware and bacterial suspensions, are most conveniently sterilised by autoclaving. The efficiency of the procedure requires regular monitoring.

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7. References

- Cousins, D.V., Francis, R.R., and Gow, B.L. (1989). Advantages of a new agar medium in the primary isolation of *Mycobacterium bovis*. *Veterinary Microbiology* 20, 89-95.
- Difco Laboratories (1953). 'Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures.' 9th Edn. (Difco Laboratories: Michigan.)
- Gallagher, J., and Horwill, D.M. (1977). A selective oleic acid albumin agar medium for the cultivation of *Mycobacterium bovis*. *Journal of Hygiene, Camb.* 79, 155-60.
- Grocott, R.G. (1955). A stain for fungi in tissue sections and smears using Gomori's Methenamine silver nitrate technics. *American Journal of Clinical Pathology* 25, 1975-9.
- Holst, E.D., Mitchison, D.A., and Radhakrishna, S. (1960). Examination of smears for tubercle bacilli by fluorescence microscopy. *Leprosy Review* 31, 110-15.
- Kantor, I.N., and Roswurm, J.D. (1978). Mycobacteria isolated from nasal secretions of tuberculin test reactor cattle. *American Journal of Veterinary Research* 39, 1233-4.
- Kircher, O. (1932). Die Leistungsfähigkeit der Tiefonkultur des Tuberkelbazillus bei Verwendung besonders geeigneter flüssiger Nährboden. *Zentralblatt für Bakteriologie. Parasitenkunde and Infektionskrankheiten* 124, 403-12.
- Lesslie, I.W. (1959). The comparison of some media for the primary isolation of *Mycobacterium tuberculosis*. *Journal Comparative Pathology* 69, 1-10.
- Lillie, R. David, Fullmer, M.F. (1976). 'Histopathologic Technic and Practical Histochemistry'. (McGraw Hill: New York.)
- Luna, L.G. (1968). 'Manual of Histologic Staining Methods of the Armed Forces.' Institute of Pathology, 3rd Edn. (McGraw Hill: New York.)

McIlroy, S.G., Neill, S.D., and McCracken, R.M. (1986). Pulmonary lesions and *Mycobacterium bovis* excretion from the respiratory tract of tuberculin reacting cattle. *Veterinary Record* 118, 718-21.

Mitchison, D.A., Allen, B.W., and Lambert, R.A. (1973). Selective media in the isolation of tubercle bacilli from tissue. *Journal of Clinical Pathology* 26, 250-2.

Mitchison, D.A., Allen, B.W., and Manickavasager, D. (1983). Selective Krichner media in the culture of specimens other than sputum for mycobacteria. *Journal of Clinical Pathology* 36, 1357-61.

Rowlett, A., and Corner, L.A. (1981). The use of a conventional counterstain with auramine fluorescent stain for acid-fast organisms, to obtain better histological contrast. *Australian Veterinary Journal* 57, 53-4.

8. Appendixes

8.1. Appendix 1 — Media for the Primary Isolation of *Mycobacterium bovis*

In a trial conducted by CSIRO (L.A. Corner, unpublished data 1989), media for primary isolation of *M. bovis* from clinical material were supplied by veterinary and medical diagnostic laboratories throughout Australia. An evaluation was made of the ability of each medium supplied to support the growth of *M. bovis*. The following media were found to be useful. The recipes given are either the only formulations currently in use, e.g. modified Middlebrook 7H11 agar and tuberculosis blood agar, or the formulation that was easiest to prepare of each successful type.

8.1.1. Lowenstein-Jensen Medium with Pyruvate

8.1.1.1. Salt solution

Potassium dihydrogen phosphate	
KH ₂ PO ₄	1.50 g
Magnesium sulfate, MgSO ₄ ·H ₂ O	0.15 g
Magnesium citrate, C ₆ H ₆ O ₇ Mg	0.375 g
L-Asparagine	2.25 g
Sodium pyruvate, C ₃ H ₃ O ₃ Na	2.00 g
Distilled water	370.0 mL

Heat to dissolve. The solution is autoclaved at 15 lb (120°C) for 15 min, cooled and stored at 4°C.

8.1.1.2. Egg fluid

The eggs should be fresh and from hens maintained on a diet free from antibiotics. The eggs are washed in a soap solution to remove surface contamination then placed in 70% ethanol for 30 min. The eggs are then dried, broken into a sterile beaker and mixed thoroughly.

8.1.1.3. Malachite green solution

Make a 2% solution of malachite green in sterile distilled water and incubate for one hour at 56°C.

8.1.1.4. Medium preparation

To 618 mL of egg fluid that has been filtered through gauze, add 370 mL of salt solution and 12 mL of malachite green solution.

8.1.1.5. Inspissation

The medium is dispensed into suitable bottles or flasks and they are heated to 85°C for 30 min or until set. They are left overnight at 37°C then stored at 4°C.

8.1.2. Stonebrink's Medium (following Lesslie, 1959)

8.1.2.1. Salt solution

C ₃ H ₃ O ₃ Na	4.20 g
KH ₂ PO ₄	1.55 g
Distilled water to	250.0 mL

Adjust the pH to 6.5 with disodium hydrogen phosphate (Na₂HPO₄). Sterilise the solution by autoclaving at 15 lb (120°C) for 15 min.

8.1.2.2. Whole egg mixture

Wash eggs in soapy water to clean the surface and rinse with tap water and dry. Place eggs in a beaker of 70% ethanol for 30 min, then air dry. Break eggs into a sterile beaker and homogenise.

8.1.2.3. Dye mixture

Crystal violet	83.0 mg
Malachite green	677.0 mg
Distilled water	83.0 mL

Sterilise the solution by steaming for 30 min.

8.1.2.4. Medium preparation

Mix solutions 8.1.2.1., 8.1.2.3. and 8.1.2.4., filter through three layers of sterile muslin, dispense into suitable containers and inspissate at 85°C for 30 min or until set. Store at 4°C.

8.1.3. Modified Middlebrook 7H11 Medium (Gallagher and Horwill, 1977)

8.1.3.1. Ingredients

Difco 7H11 agar (without glycerol)	37.8 g
Malachite green	0.005 g
C ₃ H ₃ O ₃ Na	7.8 g
Distilled water	to 1.8 L
50% glucose in citric acid (see 8.1.3.4.)	8.0 mL
Oleic acid-albumin complex (see 8.1.3.5.)	200.0 mL
Sterile bovine serum	200.0 mL
Lysed sheep red blood cells (see 8.1.3.3.)	10.0 mL

8.1.3.2. Method

Mix 7H11 agar, malachite green and sodium pyruvate with distilled water for at least one hour. Sterilise by autoclaving at 15 lb (120°C) for 20 min. Cool to 56°C and add aseptically the 50% glucose solution, OA complex, bovine serum and lysed red blood cells. Mix well and dispense.

8.1.3.3. Lysed sheep red blood cells

Aseptically mix 5 mL of sterile distilled water with 5 mL of whole sheep blood. Ethylene diaminetetraacetic acid (EDTA), heparin or citrate may be used as the anticoagulant.

8.1.3.4. 50% Glucose solution in citric acid

Dissolve 250 g dextrose in warm water and add 5 mL of 10% citric acid (C₆H₈O₇). Add distilled water to 500 mL and sterilise by filtering. Store at 4°C. Use 2 mL of this solution to 450 mL of 7H11 agar base.

8.1.3.5. Oleic acid–albumin complex

Dissolve 0.6 mL oleic acid in 50 mL of 0.05 mol/L sodium hydroxide. Prepare at 5% solution of bovine albumin fraction V by dissolving 47.5 g in 950 mL of sterile normal saline (0.85% sodium chloride). Add 50 mL of sodium oleate to 950 mL of bovine albumin fraction V solution.

Adjust the pH to 6.8. Filter, placed in water bath at 56°C for 30 min. Dispense in 50 mL aliquots and store at 4°C.

8.1.4.1. Ingredients

TB broth base (without Tween 80) (Difco)	11.0 g
C ₃ H ₃ O ₃ Na	2.0 g
L-asparagine	2.5 g
Agar	15.0 g
Bovine blood (sterile defibrinated)	83.0 mL
Penicillin (20 000 units/mL)	1.4 mL
Distilled water	916.0 mL

8.1.4.2. Method

Prepare TB broth base and steam for 30 min to dissolve. Autoclave at 15 lb for 20 min and cool to 50°C. Aseptically add the bovine blood and penicillin. Dispense.

TB broth base without Tween 80, if not available commercially, can be prepared in the laboratory according to the following formulation (Difco Laboratories, 1953).

Bacto Yeast extract	2.0 g
Proteose Peptone No. 3 (Difco)	2.0 g
Na ₂ HPO ₄	2.5 g
KH ₂ PO ₄	1.0 g
Trisodium citrate, C ₆ H ₅ Na ₃ O ₇	1.5 g
MgSO ₄	0.6 g

8.1.5. Kirchner Broth (Kirchner 1932, as modified by B.Gow, pers. comm. 1987)

8.1.5.1. Base

Na ₂ HPO ₄	171.0 g
KH ₂ PO ₄	18.0 g
MgSO ₄	5.4 g
C ₆ H ₅ Na ₃ O ₇	22.5 g
L-asparagine	45.0 g
Agar	6.75 g
Glycerol	180.0 mL
Distilled water	9.0 L

Dissolve all but the agar in 8.0 L of warm water. Dissolve the agar in 1.0 L of boiling water, then combine the solutions. Dispense in 1620 mL aliquots. Autoclave at 10 lb (115°C) for 20 min, cool and store at 4°C.

8.1.5.2. Preparation

Kirchner base	620.0 mL
C ₃ H ₃ O ₃ Na	3.6 g

Steam for 60 min. Cool to 56°C.

Aseptically add:

Glucose 20% (sterile)	9.0 mL
Serum (inactivated, sterile)	190.0 mL
Stock penicillin (20 000 units/mL)	2.25 mL

Dispense, incubate at 37°C. Store at 4°C.

8.1.6. Selective Kirchner Broth (Mitchison *et al.*, 1983)

8.1.6.1. Base

Na ₂ HPO ₄	19.0 g
KH ₂ PO ₄	2.0 g
MgSO ₄	0.6 g
C ₆ H ₅ Na ₃ O ₇	2.5 g
L-asparagine	5.0 g
Glycerol	20.0 mL
Casein hydrolysate, (Oxoid Tryptone L42)	0.5 g
Phenol red	0.012 g
Distilled water	1.0 L
pH	6.9–7.2

Distribute into 8 mL volumes and autoclave at 115°C for 10 min.

8.1.7. Antibiotic Supplement Polymyxin – Amphotericin–Carbenicillin–Trimethoprim (PACT)

Polymyxin B sulfate (1 mg=7900 units)	0.025 g
Trimethoprim lactate	0.01 g
Carbenicillin	0.05 g
Distilled water	100.0 mL
Filter sterilise	100.0 mL

Reconstitute a vial of injectable Amphotericin B (Squibb) to give a solution of 5 mg/mL. Add 0.2 mL of Amphotericin solution to sterile PACT. Distribute as 10 mL volumes and store frozen.

8.1.6.3. Preparation

For use: to a volume of PACT add an equal volume of sterile inactivated horse or calf serum. Mix and add 2.0 mL of the mixture to 8.0 mL of selective Kirchner base.

8.1.7. Antibiotic Supplements

8.1.7.1. Mitchison *et al.* (1973)

Antibiotics may be added to the medium to make it more selective for *M. bovis*. Those recommended are:

Polymyxin B sulfate	200 units/mL
Amphotericin B	10 mg/mL
Carbenicillin	100 mg/mL
Trimethoprim	10 mg/mL

8.1.7.2. P. Anagyros (pers. comm. 1987).

This antibiotic combination has been used in the preparation of selective media for the isolation of *M. tuberculosis* and *M. bovis*.

Nalidixic acid	35 mg/L
Vancomycin	20 mg/L
Polymyxin B sulfate	800 IU/L
Natamycin	100 mg/L

8.2. Appendix 2 — Staining Procedure

8.2.1. Ziehl–Neelsen Method for Bacterial Smears

There are several variations of this staining procedure found in various publications. They differ in the concentration of fuchsin used, the composition of the decolorising solution and the counterstain used. The method below gives reliable results for bacterial smears.

8.2.1.1. Reagents

8.2.1.1.1. Carbol fuchsin

Basic fuchsin	0.3 g
Ethanol 95%	10.0 mL
Phenol	5.0 g
Distilled water	95.0 mL

8.2.1.1.2. Acid alcohol

C ₂ H ₅ OH (95%)	97.0 mL
Concentrated HCl	3.0 mL

8.2.1.1.3. Counterstain

Malachite green or methylene blue	1.0 g
Distilled water	100.0 mL

8.2.1.1.4. Alkaline tap water

Alkaline tap water increases the intensity of the counterstain. Add a small amount of alkali e.g., 1 mL of 1 mol/L sodium hydroxide to 500 mL of tap water.

8.2.2.2. Procedure

- (a) Prepare smears and air dry for 10 min under ultraviolet (UV) light, in a biohazard cabinet.
- (b) Heat fix for two hours at 60–70°C.
- (c) Place slides on a rack such that their edges are not touching.
- (d) Flood each slide with carbol fuchsin.
- (e) Heat each slide gently until a small amount of steam rises. Do not boil. Leave for 5–10 min.
- (f) Rinse with tap water, then with the acid-alcohol for one to two minutes.
- (g) Rinse thoroughly with tap water.
- (h) Flood with counterstain. Leave for one to two minutes.
- (i) Rinse with alkaline tap water. If necessary additional rinsing can be done with tap water.
- (j) Air dry.

8.2.2.3. Results

Acid-fast bacteria stain red. Other organisms and organic material stain green or blue depending on the counterstain used.

8.2.2. Auramine-O Stain for Bacterial Smears.
(Holst *et al.*, 1960)

8.2.2.1. Reagents

8.2.2.1.1. Auramine-phenol

Phenol crystals	60.0 g
Auramine	6.0 g
Distilled water	2 L

Dissolve phenol in distilled water. Warm to 40–42°C. Add Auramine-O and shake vigorously. Stand overnight in the dark. Filter and store in a dark bottle.

8.2.2.1.2. Acid alcohol

NaCl	40.0 g
Concentrated HCl	40.0 mL
Ethanol (95%)	2920.0 mL
Distilled water	1040.0 mL

Dissolve sodium chloride in distilled water. Add hydrochloric acid. Add the ethanol.

8.2.2.1.3. Quenching solution

Potassium permanganate, KMnO ₄	2.0 g
Distilled water	2 L

Add the potassium permanganate to the water and store in a dark bottle.

8.2.2.2. Procedure

- (a) Fix smear at 60–70°C.
- (b) Stain for eight minutes with Auramine-phenol without heating.
- (c) Wash with distilled water.
- (d) Decolorise with acid-alcohol for two minutes.
- (e) Wash with distilled water
- (f) Quench (i.e. remove background fluorescence) with potassium permanganate solution for 30 s.

8.2.2.3. Results

Acid-fast bacteria are bright yellow on a dark background.

8.2.3. Auramine-O Stain for Tissue Sections

An auramine-O stain for acid-fast bacteria in tissue sections using a conventional counterstain allows for easy identification of the acid-fast bacteria and also the ability to ascertain the tissue architecture (Rowlatt and Corner, 1981).

The Ziehl-Neelsen technique has been the traditional method for the demonstration of acid-fast bacteria in tissue. One difficulty in using this stain is that high magnifications are required. When using a fluorochrome to demonstrate acid-fast bacteria lower magnifications can be employed and therefore the whole section can be examined in a shorter time.

The disadvantage of using potassium permanganate or ferric chloride to quench background fluorescence is that tissue structures cannot be identified. In order to overcome this difficulty, a conventional counterstain, basic fuchsin, together with UV light was used to enable the bacteria to be identified against a background where cells and tissue structures are sufficiently visible to allow identification. The intensity of the counterstain is considerably below that of the bacteria.

8.2.3.1. Reagents

8.2.3.1.1. Auramine-phenol solution

Auramine O	3.0 g
Glycerol	150.0 mL
Ethanol	20.0 mL
Phenol	20.0 g
Distilled water	100.0 mL

Dissolve the auramine in the water and glycerol mixture by warming to 45°C. Add the ethanol and then the phenol. Do not filter. The solution keeps for a least three months.

8.2.3.1.2. Acid alcohol solution

Ethanol	70.0 mL
Conc. HCl	0.5 mL
Distilled water	29.5 mL

8.2.3.2. Procedure

Tissues are fixed, sectioned and rehydrated as normal (Lillie and Fullmer, 1976).

- (a) Stain for 15 min at 60°C in phenol–auramine solution.
- (b) Remove the slides from the stain; wash in running tap water to remove free auramine.
- (c) Differentiate in 0.5% HCl in 70% ethanol, one minute in each of two baths, with intermittent agitation of the slides.
- (d) Wash for one to two minutes in running tap water.
- (e) Counterstain with 0.01% aqueous basic fuchsin for 30 s.
- (f) Rinse in running water, dehydrate and differentiate the counterstain in three changes of absolute ethanol to a light pink shade when viewed macroscopically.

- (g) Clear in two changes of xylene [$C_6H_4(CH_3)_2$], mount in non-fluorescing medium.
- (h) Examine separately with transmitted white light or incident UV light using a 436 nm excitation filter and 520 nm barrier filter and x40 or x63 objectives.

8.2.3.3. Results

8.2.3.3.1. By white light. Tissue in shades of red and pink.

8.2.3.3.2. By UV light. Acid-fast bacteria appear bright yellow on a dull red–orange or yellow background, the colour depending on the tissue components.

ATTACHMENT VII

SUMMARY

The Australian National Quality Assurance Program for veterinary diagnostic testing aims to establish quality assurance in 21 laboratories in Australia and New Zealand. Not all of the participating laboratories perform all of the tests.

This is achieved by designating national reference antisera for each test and attempting to ensure standard methods are used in all laboratories in line with the Australian Standard Diagnostic Techniques for Animal Diseases. Each laboratory is assessed on the result it provides for the reference standards, plus a number of unknown antisera for each test. The results reported for the unknown antisera are statistically analysed.

In Phase 1, which was completed in March 1991, four tests were evaluated. In Phase 2, completed in September 1992, the same four tests and eight additional tests were evaluated. In Phase 3, completed in October 1992, the twelve tests evaluated in Phase 2 and an additional 13 tests were evaluated. Phase 4, completed in May 1994, involved the 25 tests examined in Phase 3 and an additional 9 tests. Phase 5, completed in January 1995, evaluated the same 34 assays as Phase 4 with the exception of the Bluetongue complement fixation test which was replaced by the *Brucella ovis* ELISA. Phase 6, 1995, evaluated the same 34 assays as Phase 5. Phase 7, 1996, increased to 35 serological assays with the inclusion of the EBL ELISA. Since Phase 4 ANQAP has extended to include non-serological quality assurance programs such as the culture and identification of *Mycobacterium bovis*. Two new quality assurance programs have been included in the annual Phase 8 ANQAP evaluation, these include Footrot gelatin gel interlaboratory testing and the culture and identification of *Mycobacterium paratuberculosis*. All three programs are included in this report. Future expansion of the program in Phase 9 testing 1998 will include interlaboratory testing of Avian Influenza AGID, Newcastle Disease Virus HI, Rabbit Calicivirus Virus Disease ELISA, Myxoma ELISA and milk EBL ELISA.

This report includes the proficiency testing results for 38 assays evaluated in Phase 8 including *M. bovis* culture, *M. paratuberculosis* culture and Footrot gelatin gel. Phase 8 testing began in January 1997 and concluded in January 1998.

The procedure for classifying results was introduced in Phase 6 and developed further in Phase 7. Phase 8 was the first evaluation in which results were formally classified based on the ANQAP quality procedure QP0010 'Endorsement of Laboratories for Export Testing through Interlaboratory Proficiency Testing via ANQAP'. Laboratories reporting acceptable results were listed on the ANQAP Endorsed List of Laboratories which was published every quarter and distributed to SCAHLS, ANQAP participants and Chief Veterinary Officers.

The following statistical breakdown can be concluded from the Phase 8 classification of results:

The total number of evaluations required during Phase 8 was 269. In 183 of these evaluations (68%), results were within the acceptable variation range (AVR) from the consensus mean values and were classified as acceptable (✓). These figures are comparable to the Phase 7 evaluation where 73% of results fell within the AVR. Those laboratories which reported results outside the AVR were required to retest. Generally, all but three laboratories reported results which fell within the AVR or one dilution of the AVR limits on retesting, and were classified as acceptable or demonstrating minor variation. Only 3 of the 269 evaluations demonstrated significant variation due to decreased sensitivity, where positive results were consistently interpreted as negative. Laboratory 3 was not endorsed for the Akabane SNT, laboratory 13 was not endorsed for the BT AGID and laboratory 14 was not endorsed for the BVD AGID. In an internal

A U S T R A L I A N N A T I O N A L Q U A L I T Y A S S U R A N C E P R O G R A M

investigation each laboratory identified the cause of the decreased sensitivity and implemented improved procedures accordingly. Two laboratories attributed the loss of sensitivity to internal reagents and one laboratory identified a discrepancy in the interpretation method between the ASDT and their laboratory procedure. All three laboratories reported acceptable results on re-endorsement testing and were subsequently included on the ANQAP Endorsed List of Laboratories for the three tests.

The SNT and MAT demonstrated the largest variation with approximately 50% of laboratories requiring retesting. Serious concerns were identified with the Aino, Akabane SNT and all three MAT. The large between laboratory variation appears to be caused by variation in methodology and reagents. These issues will be addressed in the review of the ASDT and an SNT review by ANQAP in Phase 9. Results submitted for the AGID demonstrated the least variation with 81% results acceptable on original testing. The ELISA demonstrated only minor variation with 72% results acceptable on original testing.

Overall only 1% of the evaluations in Phase 8 were not initially endorsed, with 99% of laboratory results classified as acceptable or demonstrating minor variation on original or retesting.

The details and summary of the quality assurance testing for *M.bovis*, *M.paratuberculosis* and Footrot Gelatin Gel are included in the final chapter of this report.

ANQAP *Mycobacterium bovis*. Interlaboratory Comparison

Background

The Quality Assurance (QA) program for culture and identification of *Mycobacterium bovis* was established as part of the responsibilities of the Australian Reference Laboratory for Bovine Tuberculosis (ARLBTB) and commenced in 1993. With decreasing numbers of *M. bovis* being isolated towards the end of the Brucellosis and Tuberculosis Eradication Campaign and the loss of experienced staff as a direct consequence of the success of the Campaign, it was considered necessary to establish a QA program to remain confident of our ability to diagnose bovine tuberculosis effectively.

The program aims to produce duplicate samples within the 12 samples supplied. The samples were designed so that one pair would contain no organisms, one pair would contain *M. spp* and three pairs would contain decreasing numbers of *M. bovis*. The remaining two samples were tissue specimens from a 1996 case of bovine tuberculosis.

Sample preparation

Tissue suspensions for spiked samples were prepared by homogenising normal lymph node tissue to a consistency that could be dispensed by pipette (~20%). Two mL of the tissue suspension was added to polycarbonate bijoux bottles and stored at -20_C.

Suspensions of *M. bovis* and *M. spp* equivalent to a McFarland No. 5 Opacity tube were prepared and subjected to a brief sonication to disperse large clumps. Ten-fold dilutions from 10⁻¹ to 10⁻⁷ were made in sterile purified water and a viable count was performed to determine the number of organisms present. One hundred microlitres of each of dilution was added to 2 mL of tissue homogenate and after vortex mixing, the samples were stored at -80_C. When results of the viable count were known, four sets of spiked samples (3 x *M. bovis*, 1 x *M. spp*) were selected for inclusion in the QA.

In addition 12 bottles of uninoculated tissue suspension (negative control) and the 12 lesioned tissue samples were included.

Prepared samples were stored for approximately 2 months awaiting the results of the viable count and a preliminary culture on one set of samples to ensure that samples were satisfactory for QA purposes.

Twelve sets of 6 samples (i.e. 12 x 6 x 2 = 144 tubes) were used. Pairs were assigned randomly.

- Samples 4 & 8 had no organisms added.
- Samples 7 & 12 had 100 uL of 10⁻² suspension of *M. spp* (~2,500 cfu) added.
- Samples 6 & 11 each consisted of a small piece of tissue cut from lesioned material from two confirmed case of tuberculosis.
- Samples 1 & 9 had 100 uL of 10⁻¹ suspension of *M. bovis* (~22,000 cfu) added.
- Samples 2 & 5 had 100 uL of 10⁻³ suspension of *M. bovis* (~220 cfu) added.
- Samples 3 & 10 had 100 uL of 10⁻⁴ suspension of *M. bovis* (~22 cfu) added.

Prior to shipment, tins were cooled to -80_C and the samples added. The packed tins each containing 12 samples were stored at -80_C until they were transported. The tins were shipped on dry ice to each of the participating laboratories by overnight courier. The submitting laboratory cultured the samples one week after sending out samples to other participating laboratories.

Results

Seven laboratories participated in the fifth inter-laboratory testing for culture and identification of *M. bovis*. Five samples prepared in duplicate and 2 samples of lesioned material were examined.

Laboratory D was supplied with 2 sets of the homogenates for treatment with 2 different decontaminants (HPC and oxalic acid). Three samples treated with oxalic acid were culture negative (both samples with lowest numbers of *M. bovis* and one with *M. spp*) but culture positive using the HPC decontamination method. No isolations were missed using HPC.

Laboratory A was supplied with two sets of the homogenates for treatment using two culture methods (HPC/conventional culture and NaOH/BACTEC). HPC/conventional culture isolated the two *M. spp* isolates but failed to detect one sample with low concentration *M. bovis*.

Laboratories B split their samples into two and used two different media (conventional solid media and BACTEC). Solid media achieved isolation in two cases where BACTEC failed.

Laboratory F split their samples into two and used two different decontaminating agents (HPC and NaOH). Contamination was evident in two of the samples treated with HPC, and in two cases isolation of *M. spp* was only achieved in the samples tested with HPC.

Laboratory G split their samples and assessed HPC at two different concentrations (0.75% and 0.075%). This laboratory had problems with media drying out the results were inconclusive with the samples supplied in June 1997. Following discussion with the ARLBTB and because a spare set of samples was held in storage at the ARLBTB, an additional set of samples was submitted to Laboratory G for testing.

Four laboratories identified mycobacteria as *M. bovis* or *M. spp*. Three laboratories recorded the isolation of acid-fast bacilli only, and sent their isolates to the ARLBTB for further identification.

The results summarised below include the composite (combined best) results in cases where two sets of samples were tested or samples were split for testing by two methods in individual laboratories (Table 1, Figure 1).

These results are for the samples sent out in June 1997 for Laboratories A-F, and for Laboratory G are for samples sent in October 1997.

Final identification is as supplied by the participating laboratory in the case where it was performed in-house, or as provided by the ARLBTB after referral.

Sample 1: (<i>M. bovis</i> 22,000 cfu)	All seven laboratories reported the isolation of <i>M. bovis</i> .
Sample 2: (<i>M. bovis</i> 220 cfu)	All seven laboratories reported the isolation of <i>M. bovis</i> .
Sample 3: (<i>M. bovis</i> 22 cfu)	Four laboratories reported the isolation of <i>M. bovis</i> . Three laboratories (B, E and G) reported this sample as negative.

<i>Sample 4:</i> (negative)	All seven laboratories reported a negative result.
<i>Sample 5:</i> (<i>M. bovis</i> 220 cfu)	Six laboratories reported the isolation of <i>M. bovis</i> . One laboratory (C) reported the sample as contaminated.
<i>Sample 6 :</i> (Lesioned material from <i>M. bovis</i> +++ clinical case)	All seven laboratories reported the isolation of <i>M. bovis</i> .
<i>Sample 7:</i> (<i>M. spp</i> 2,500 cfu)	All 7 laboratories reported the isolation of <i>M. spp</i> .
<i>Sample 8:</i> (negative)	Six laboratories reported a negative result and one laboratory (G) reported isolation of <i>M. spp</i> .
<i>Sample 9:</i> (<i>M. bovis</i> 22,000 cfu)	Six laboratories reported the isolation of <i>M. bovis</i> . One laboratory (G) reported the isolation of <i>M. spp</i> .
<i>Sample 10:</i> (<i>M. bovis</i> 22 cfu)	Two laboratories (D and G) reported the isolation of <i>M. bovis</i> . Five laboratories reported this sample as negative.
<i>Sample 11:</i> (<i>M. bovis</i> infected tissue)	All seven laboratories reported the isolation of <i>M. bovis</i> .
<i>Sample 12:</i> (<i>M. spp</i> 2,500 cfu)	Six laboratories reported the isolation of <i>M. spp</i> . One laboratory (B) reported this sample as negative.

An assessment of each laboratory is documented in Table 2.

Conclusions:

- From the information supplied in the paperwork, most laboratories appeared to be using techniques within the guidelines of the Australian Standard Diagnostic Techniques for Animal Diseases.
- There was excellent agreement between laboratories in the negative samples.
- None of the laboratories reported any serious problems with contamination.
- In both laboratories where the BACTEC system was used, it was slightly less successful in some cases with lower numbers of organism.
- Laboratory D performed very well, reporting the same results for all of the duplicates, and detecting mycobacteria at the lowest level (22 cfu). The same level of sensitivity was not recorded when Oxalic acid was used as a decontaminating agent.
- Laboratories A and F also performed well, reporting good sensitivity and accuracy, with the same results for all of the duplicates with the exception of one of the lowest dilutions of *M. Bovis*.

- The results of Laboratories C and E were acceptable. Laboratory C missed one sample with the lowest level of *M. bovis* (the duplicate to this sample yielded a single colony) and one sample was contaminated. Laboratory E was unable to isolate from the sample with the lowest dilutions of *M. bovis*, but otherwise their performance was good.
- Laboratory G experienced problems when testing the samples sent in June 1997 it was unable to provide conclusive results on this set of samples. Following investigation and discussion between staff in Laboratory G and the ARLBTB, it was agreed that the situation was most likely due to media problems. The batch of media used [supplied from a local hospital and not subject to quality control (QC)] dried out after approximately 3 weeks incubation.

Laboratory G - results of testing samples re-supplied in October 1997

- Laboratory G performed mycobacteria culture on a second set of samples supplied on 23 October 1997, using both local and ARLBTB supplied media. The ARLBTB supplied media achieved slightly better results than locally prepared media did.
- On re-test, the composite results of Laboratory G was generally satisfactory although it was noted that *M. spp* was isolated from one sample that was culture negative in all other laboratories (sample 8) and from one sample from which other laboratories isolated *M. bovis* (sample 9).
- The fact that Laboratory G experienced problems with their media highlights the fact that problems with culture media can occur. Other laboratories have experienced some similar problems previously.

Recommendation:

Laboratories should only use media that has been quality controlled and proven to sustain growth of *M. bovis*. This QC should be provided by the media suppliers or can be performed 'in-house' on each new batch supplied and should be completed before the QA samples arrive for testing.

Table 1: Composite results of laboratories A-F for samples supplied in June 1997 and for laboratory G on samples re-supplied in October 1997.

	A	B #	C	D	E	F #	G #Oct samples
1	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>
2	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>
3	<i>M. bovis</i>	Negative	<i>M. bovis</i> *	<i>M. bovis</i>	Negative	<i>M. bovis</i>	Negative
4	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	<i>M. bovis</i>	<i>M. bovis</i>	contaminated?	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>
6	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>
7	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>
8	Negative	Negative	Negative	Negative	Negative	Negative	<i>M. spp</i>
9	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. spp</i>
10	Negative	Negative	Negative	<i>M. bovis</i>	Negative	Negative	<i>M. bovis</i>
11	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>	<i>M. bovis</i>
12	<i>M. spp</i>	Negative	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>	<i>M. spp</i>

* single colony

referred for identification

^ not sent for ID, ZN results only

Table 2: Final assessment of laboratories in culture of *M. bovis* during 1997.

Laboratory	A	B	C	D	E	F	G
Rating	Ā	Ā	Ā	Ā	Ā	Ā	M

Ā results submitted by this Laboratory were satisfactory

M results submitted by this Laboratory demonstrate minor variation from the expected results. The results are acceptable, however the minor variation is worthy of note.

Figure 1. Graphical representation of results on duplicate samples sent to participants in June 1997, given as percent acceptable (0, 50 or 100%).

