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Bovine Tuberculosis

Immunodiagnosis

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Contents

1. Introduction	3
2. Single intradermal caudal fold tuberculin test	3
2.1. Technique	3
2.2. Interpretation	3
3. Comparative intradermal tuberculin test	4
3.1. Technique	4
3.2. Interpretation	4
4. Interferon- γ assay	5
4.1. Collection of blood samples	5
4.2. Transport and storage of blood samples	5
4.3. Incubation of whole blood with tuberculin	6
4.4. Harvesting of plasma after incubation	6
4.5. Interferon- γ enzyme immunoassay	6
4.6. Interpretation of results	7
5. Repeat testing	8
6. References	8
7. Appendixes	8
7.1. Appendix 1 — Equipment required for the interferon- γ whole blood culture	8
7.2. Appendix 2 — Equipment required for harvesting plasma samples	8
7.3. Appendix 3 — Equipment required for the interferon- γ enzyme immunoassay	8

Bovine Tuberculosis

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 (Rothelet *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 McLintock syringe) and short needles (3–4 mm) of 26 gauge or finer should be used for injections. McLintock 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 animal's 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 animal's 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

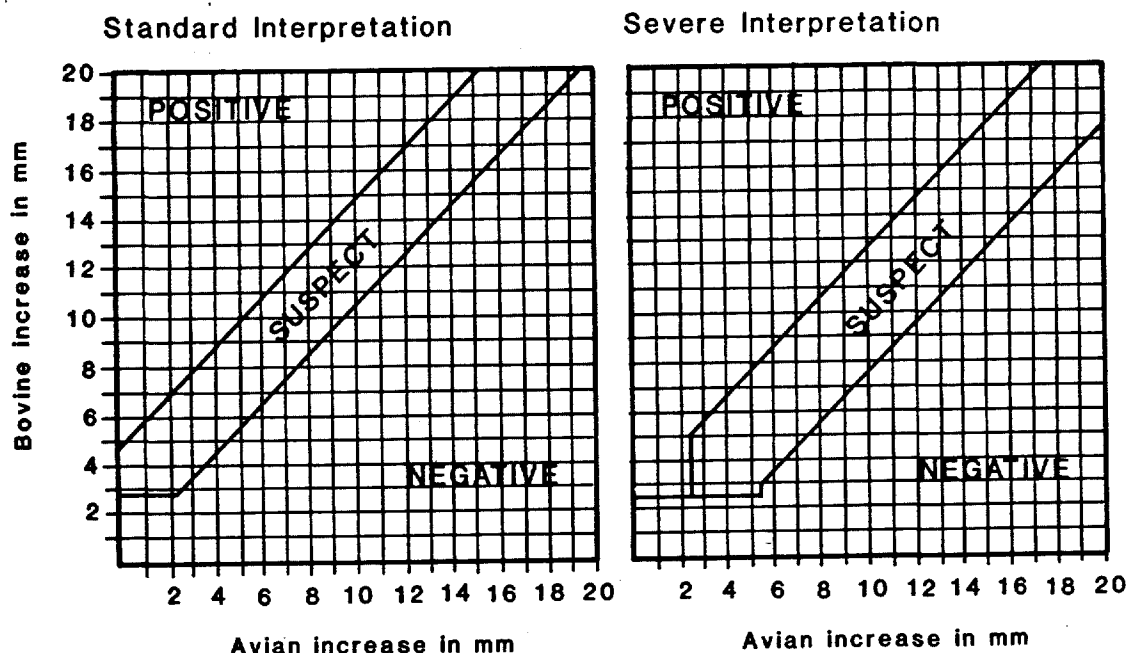


Figure 1. Interpretation of the comparative intradermal tuberculin test.

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 Combipipette 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
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
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

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7. Appendixes

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

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

7.2. Appendix 2 — Equipment Required for Harvesting Plasma Samples

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

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

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