CHAPTER 2.4.7.

BOVINE TUBERCULOSIS

SUMMARY

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by Mycobacterium bovis. *In a large number of countries bovine tuberculosis is a major infectious disease among cattle, other domesticated animals, and certain wildlife populations. Transmission to humans constitutes a public health problem.*

Aerosol exposure to M. bovis *is considered to be the most frequent route of infection of cattle, but infection by ingestion of contaminated material also occurs. After infection, nonvascular nodular granulomas known as tubercles may develop. Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, on serous membranes, and in other organs.*

Bovine tuberculosis infection in cattle is usually diagnosed in the live animal on the basis of delayed hypersensitivity reactions. Infection is often subclinical; when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques. Rapid nucleic acid methodologies, such as the polymerase chain reaction (*PCR*), *may also be used although these are demanding techniques and should only be used when appropriately validated. Traditional mycobacterial culture remains the gold standard method for routine confirmation of infection.*

Identification of the agent: *Bacteriological examinations may consist of the demonstration of acid-fast bacilli by microscopic examination, which provides presumptive confirmation. The isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA techniques, such as PCR, confirms infection. Animal inoculation, which has been used in the past for confirming infection with* M. bovis, *is now rarely used because of animal welfare considerations.*

Delayed hypersensitivity test: *This test is the standard method for detection of bovine tuberculosis. It involves measuring skin thickness, injecting bovine tuberculin intradermally into the measured area and measuring any subsequent swelling at the site of injection 72 hours later.*

The comparative intradermal tuberculin test with bovine and avian tuberculin is used mainly to differentiate between animals infected with M. bovis *and those sensitised to tuberculin due to exposure to other mycobacteria or related genera.*

The decision to use the single or comparative test generally depends on the prevalence of tuberculosis infection and on the level of environmental exposure to the other sensitising organisms.

Due to their higher specificity and easier standardisation, purified protein derivative (*PPD*) *products have replaced heat-concentrated synthetic medium tuberculins. The recommended dose of bovine PPD in cattle is at least 2000 International Units* (*IU*) *and in the comparative tuberculin test, the doses should be no lower than 2000 IU each. The reactions are interpreted on the basis of the test method used.*

Blood-based laboratory tests: *Diagnostic blood tests are now available, e.g. the gamma-interferon assay, which uses an enzyme-linked immunosorbent assay* (*ELISA*) *as the detection method for interferon, the lymphocyte proliferation assay, which detects cell-mediated immune responses, and the indirect ELISA, which detects antibody responses. The logistics and laboratory execution of some of these tests may be a limiting factor. The use of blood-based assays can be*

advantageous, especially with intractable cattle, zoo animals and wildlife, although interpretation of the test may be hampered by lack of data for some species. Information on the use of various diagnostic tests in animal species other than bovine is provided in a recent review by Cousins & Florisson (*15*).

Requirements for vaccines and diagnostic biologicals: *Vaccines are being developed and evaluated for use in bovine and wildlife species, but at this time are not routinely administered as they may compromise the use of the tuberculin skin test and other immunological tests to detect infected animals. There are standard methods for the production of bovine PPD tuberculins. PPD, used for performing the tests specified, should be prepared in accordance with the World Health Organization requirements and should conform to these requirements with respect to source materials, production methods and precautions, added substances, freedom from contamination, identity, safety, potency, specificity and freedom from sensitising effect. The bioassays for biological activity are of particular importance, and the potency should be expressed in IUs.*

A. INTRODUCTION

Mycobacterium bovis is a zoonotic organism and should be treated as a risk/hazard group III organism with appropriate precautions to prevent human infection occurring.

Bovine tuberculosis is an infectious disease caused by *M. bovis* that affects cattle, other domesticated animals and certain free or captive wildlife species. It is usually characterised by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume a more progressive course. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum.

It should be noted that other members of the *M. tuberculosis* complex, previously considered to be *M. bovis,* have been accepted as new species despite identical 16s RNA sequences and over 99.9% identity of their genome sequences. These include *M. caprae* (3) (in some countries considered to be a primary pathogen of goats) and *M. pinnipedii* (14), a pathogen of fur seals and sea lions. These two new species are known to be zoonotic. In central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (49). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis. In countries with tuberculosis eradication programmes, clinical evidence of tuberculosis in cattle is seldom encountered because the intradermal tuberculin test enables presumptive diagnosis and elimination of infected animals before signs appear. Prior to the national tuberculosis eradication campaigns, however, clinical signs associated with tuberculosis were commonly observed (13).

In many cases, the course of the infection is chronic and signs may be lacking, even in advanced cases when many organs may be involved. When present, clinical signs vary; lung involvement may be manifested by a cough, which can be induced by changes in temperature or manual pressure on the trachea. Dyspnoea and other signs of low-grade pneumonia are also evidence of lung involvement.

In advanced cases, lymph nodes are often greatly enlarged and may obstruct air passages, the alimentary tract, or blood vessels. Lymph nodes of the head and neck may become visibly affected and sometimes rupture and drain. Involvement of the digestive tract is manifested by intermittent diarrhoea and constipation in some instances. Extreme emaciation and acute respiratory distress may occur during the terminal stages of tuberculosis. Lesions involving the female genitalia may occur. Male genitalia are seldom involved.

At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected. In addition, the lung, liver, spleen and the surfaces of body cavities are commonly affected. Early nodular pulmonary lesions can often be detected by palpation. The lesions are usually non-odoriferous. Other anatomical sites can be infected and should be examined.

Macroscopically, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be purulent. The appearance may be more purulent in cervids and camelids. Some nontuberculous granulomas may be indistinguishable macroscopically from tuberculous granulomas.

The caseous centre is usually dry, firm, and covered with a fibrous connective capsule of varying thickness. Lesion size ranges from small enough to be missed by the unaided eye, to involvement of the greater part of an organ. Serial sectioning of organs and tissues may be required to detect the small lesions contained within the tissue. Histologically, lesions caused by *M. bovis* are often paucibacillary (having few organisms) and the absence

of acid-fast organisms does not exclude tuberculosis in lymphadenitis of unknown aetiology. In cervidae and some exotic species, tuberculosis should be considered when thin-walled purulent abscesses are observed in the absence of specific aetiology.

Mycobacterium bovis has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully characterised. The incidence of pulmonary tuberculosis caused by *M. bovis* is higher in farm and slaughterhouse workers than in urban inhabitants. The transmission of *M. bovis* to humans via milk and its products is eliminated by the pasteurisation of milk. One of the results of bovine tuberculosis eradication programmes has been a reduction in disease and death caused by bovine tuberculosis in the human population.

Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and nondomesticated animals. Isolations have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, South American camelids, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (18, 47).

Bovine tuberculosis in wildlife was first reported in 1929 in greater kudu (*Tragelaphus strepsiceros*) and common duiker (*Sylvicapra grimmi*) in South Africa and by the 1940s, the disease was found to be endemic in greater kudu. In 1982 in Uganda, a prevalence of 10% in African buffalo and 9% in warthog (*Phacochoerus aethiopicus*) was found, and in Zambia, *M. bovis* infection has been reported in Kafue lechwe (*Kobus leche kafuensis*) and in a single eland (*Traurotragus oryx*). An outbreak of tuberculosis in wild olive baboons (*Papio cynocephalus anubis*) was reported in Kenya. *Mycobacterium bovis* infection has also been diagnosed in African buffalo in the Kruger National Park in South Africa (4), and more recently spill over to other species such as chacma baboon (*Papio ursinus*), lion (*Panthera leo*) and cheetah (*Acynonyx jubatus*) as well as greater kudu has occurred.

The rigorous application of tuberculin testing and culling of reactor cattle has eliminated *M. bovis* infection from farmed bovine populations in some countries, but this strategy has not been universally successful. Extensive investigations of sporadic *M. bovis* reoccurrence have shown that wildlife reservoirs exist in some countries and can act as a source of infection for cattle, deer and other livestock. The risk that these reservoirs of infection constitute for domestic animals and humans is quite variable depending on the specific epidemiological situation for the species and the environment (12, 42). The detection of infection in a wildlife population requires bacteriological investigation or the use of a valid testing method for the species involved (the tuberculin test is not effective in all species) together with epidemiological analysis of information. The badger (*Meles meles*) in the United Kingdom (57) and the Republic of Ireland (47), wild boar (*Sus scrofa*) in Spain (43), the brush-tail possum (*Trichosurus vulpecula*) in New Zealand (2), and several wild living species in Africa have been shown to be capable of maintaining *M. bovis* infection. Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population. The use of vaccination to control the disease in some species continues to be investigated.

Mycobacterium bovis has been isolated from farmed and free-living cervidae. The disease may be subacute or chronic, with a variable rate of progression. A small number of animals may become severely affected within a few months of infection, while others may take several years to develop clinical signs, which are related to lesions in the animal. The lesions produced may resemble those found in cattle (caseating granulomatous inflammation that is often mineralised). The lesions may take the form of thin-walled abscesses with little calcification and containing purulent material. Thin-walled abscesses have also been observed in llamas. In cervids, tuberculosis should be considered when abscess-like lesions of no known aetiology are observed. The lymph nodes affected are usually those of the head and thorax. The mesenteric lymph nodes may be affected – large abscesses may be found at this site. The distribution of lesions will depend on the infecting dose, route of infection and the incubation period before examination.

The tuberculin test can be used in farmed deer. The test must be carried out on the side of the neck. To obtain results that are valid, the hair should be clipped around the injection site, there should be accurate intradermal injection of the tuberculin and careful pre- and post-inoculation skin thickness measurement should be made using callipers (8).

B. DIAGNOSTIC TECHNIQUES

When diagnostic techniques are used within official TB control or eradication programmes, it is recommended the Veterinary Administration authorises:

- The diagnostic test(s);
- Laboratories performing the tests; and
- Those persons applying diagnostic techniques to animals, i.e. skin tests.

1. Identification of the agent

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed. For this reason, its diagnosis in individual animals and an eradication programme were not possible prior to the development of tuberculin by Koch in 1890. Tuberculin, a concentrated sterile culture filtrate of tubercle bacilli grown on glycerinated beef broth and, more recently, on synthetic media, provides a means of detecting the disease.

Immunological responses to *M. bovis* infections in cattle continue to be studied in attempts to develop improved or alternative diagnostic methods, as skin testing sometimes has practical drawbacks. The gamma interferon test is increasingly being used as a diagnostic blood test for tuberculosis in cattle and for other animals (e.g. goat, buffalo) and is available commercially. The lymphocyte proliferation test and the IgG_1 enzyme-linked immunosorbent assay (ELISA) have proven to be useful as ancillary serial (to enhance specificity) and parallel (to enhance sensitivity) tests in farmed red deer.

The presence of *M. bovis* in clinical and post-mortem specimens may be demonstrated by examination of stained smears or tissue sections and confirmed by cultivation of the organism on primary isolation medium. Collection containers should be clean and preferably sterile (use of sample containers that are contaminated by environmental mycobacteria may result in the failure to identify *M. bovis* infection due to the rapid growth of the environmental mycobacteria); where feasible, single-use plastic, disposable containers, 50 ml in capacity, may be used for a variety of specimen types. Specimens that are to be sent to the laboratory must be cushioned and sealed to prevent leakage, and properly packaged to withstand breakage or crushing in transit. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed. The requirements are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens. Prompt delivery of specimens to the laboratory greatly enhances the chances of cultural recovery of *M. bovis*. If delays in delivery are anticipated, specimens should be refrigerated or frozen to retard the growth of contaminants and to preserve the mycobacteria. In warm ambient conditions, when refrigeration is not possible, boric acid may be added (0.5% [w/v] final concentration) as a bacteriostatic agent, but only for limited periods, no longer than 1 week.

Precautions should be taken to prevent infection of laboratory personnel (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). All procedures involving culture should be performed in a biological safety cabinet.

a) Microscopic examination

Mycobacterium bovis can be demonstrated microscopically on direct smears from clinical samples and on prepared tissue materials. The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl–Neelsen stain, but a fluorescent acid-fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results. The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, the presence of acid-fast organisms in histological sections may not be detected, although *M. bovis* can be isolated in culture. However, large numbers of acid-fast organisms are seen in lesions in primates, felids, mustelids (badgers) and marsupials (brush-tailed possums).

b) Culture

To process specimens for culture, the tissue is first homogenised using a mortar and pestle, stomacher or blender, followed by decontamination with either detergent (such as 0.375–0.75% hexadecylpyridinium-chloride [HPC]), an alkali (2–4% sodium hydroxide) or an acid (5% oxalic acid). The alkali or acid mixture is shaken for 10–15 minutes at room temperature and then neutralised. Neutralisation is not required when using HPC. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination. It is recommended that, as a minimum, pooled lymph node samples from the head and thorax be cultured when no visible lesions are detected in tuberculin or interferon test positive animals at post-mortem examination.

For primary isolation, the sediment is usually inoculated on to a set of solid egg-based media, such as Lowenstein–Jensen, Coletsos base or Stonebrinks; these media should contain either pyruvate or pyruvate and glycerol. An agar-based medium such as Middlebrook 7H10 or 7H11 or blood based agar medium (16) may also be used.

Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO_2. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at intervals during the incubation period. When growth is visible, smears are prepared and stained by

the Ziehl–Neelsen technique. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used.

Liquid culture systems are used routinely in some hospital and veterinary laboratories; in these systems growth is measured by radiometric or fluorometric means.

If gross contamination of culture media occurs, the culture process should be repeated using the retained inocula with an alternative decontaminating agent. The limiting factor in isolation is often the poor quality of the samples submitted and every effort should be made to insure that the laboratory receives good quality samples.

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis;* however every isolate needs to be confirmed. It is necessary to distinguish *M. bovis* from the other members of the 'tuberculosis complex', i.e. *M. tuberculosis* (the primary cause of tuberculosis in humans), *M. africanum* (occupies an intermediate phenotypic position between *M. tuberculosis* and *M. bovis*), *M. microti* (the 'vole bacillus', a rarely encountered organism), *M. pinnipedii* and *M. caprae*.

Mycobacterium tuberculosis may infect cattle and sensitise cattle to bovine tuberculin without causing typical lesions. Sometimes *M. avium* or other environmental mycobacteria may be isolated from tuberculosis-like lesions in cattle. In such cases, a careful identification is needed, and a mixed infection with *M. bovis* should be excluded.

Isolates can be identified by determining traditional cultural and biochemical properties. On a suitable pyruvate-based solid medium, colonies of *M. bovis* are smooth and off-white (buff) in colour. The organism grows slowly at 37°C, but does not grow at 22°C or 45°C. *Mycobacterium bovis* is sensitive to thiophen-2-carboxylic acid hydrazide (TCH) and to isonicotinic acid hydrazide (INH). This can be tested for by growth on 7H10/7H11 Middlebrook agar medium or on egg-containing media. The egg medium should be prepared without pyruvate because it inhibits INH and could have a similar effect on TCH (which is an analogue of INH) and thus give false-positive (resistant) results. *Mycobacterium bovis* strains are also sensitive to para-amino salicylic acid and streptomycin. Effective drug concentrations are different for egg-based and agar-based media. Results for niacin production and nitrate reduction are negative in *M. bovis*. In the amidase test, *M. bovis* is positive for urease and negative for nicotinamidase and pyrazinamidase. It is a microaerophillic and nonchromogenic bacterium.

c) Nucleic acid recognition methods

Rapid identification of isolates to the level of *M. tuberculosis* complex can be made by Gen Probe TB complex DNA probe or polymerase chain reaction (PCR) targeting 16S–23S rRNA, the insertion sequences IS*6110* and IS*1081*, and genes coding for *M.-tuberculosis*-complex-specific proteins, such as MPB70 and the 38 kDa antigen b have been used. Specific identification of an isolate as *M. bovis* can be made using PCR targeting a mutation at nucleotide positions 285 in the *oxy*R gene,169 in the *pncA* gene, 675/756/1311/1410 and 1450 of the *gyr*B gene and presence/absence of RDs (Regions of Difference) (21, 35, 44, 48). Alternatively molecular typing techniques, such as spoligotyping will identify *M. bovis* isolates and provide some molecular-typing information on the isolate that is of epidemiological value (36).

PCR has been widely evaluated for the detection of *M. tuberculosis* complex in clinical samples (mainly sputum) in human patients and has recently been used for the diagnosis of tuberculosis in animals. A number of commercially available kits and various 'in-house' methods have been evaluated for the detection of the *M. tuberculosis* complex in fresh and fixed tissues. Various primers have been used, as described above. Amplification products have been analysed by hybridisation with probes or by gel electrophoresis. Commercial kits and the in-house methods, in fresh, frozen or boric acid-preserved tissues, have shown variable and less than satisfactory results in interlaboratory comparisons (45). False-positive and false-negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of this test. Variability in results has been attributed to the low copy number of the target sequence per bacillus combined with a low number of bacilli. Variability has also been attributed to decontamination methods, DNA extraction procedures, techniques for the elimination of polymerase enzyme inhibitors, internal and external controls and procedures for the prevention of cross-contamination. Improvement in the reliability of PCR as a practical test for the detection of *M. tuberculosis* complex in fresh clinical specimens will require the development of standardised and robust procedures. Cross contamination is the greatest problem with this type of application and this is why proper controls have to be set up with each amplification. However, PCR is now being used on a routine basis in some laboratories to detect the *M. tuberculosis* group in paraffin-embedded tissues (41, 40). Although direct PCR can produce a rapid result, it is recommended that culture be used in parallel to confirm a viable *M. bovis* infection.

A variety of DNA-fingerprinting techniques has been developed to distinguish the *M. tuberculosis* complex isolates for epidemiological purposes. These methods can distinguish between different strains of *M. bovis*

and will enable patterns of origin, transmission and spread of *M. bovis* to be described (19, 20). The most widely used method is spoligotyping (from 'spacer oligotyping'), which allows the differentiation of strains inside each species belonging to the *M. tuberculosis* complex, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (34, 36). The use of a standard nomenclature for the spoligotypes according to the database Mbovis.org (http://www.mbovis.org) is encouraged to allow international comparison of profiles.

Other techniques include restriction endonuclease analysis (REA) and restriction fragment length polymorphism (RFLP) using IS*6110* probe (especially where there are >3–4 copies of IS*6110* in the isolate), the direct repeat (DR) region probe, the PGRS (polymorphic GC repeat sequence) probe (54) and the pUCD probes (46). The mycobacterial interspersed repetitive units (MIRU)-variable number tandem repeat (VNTR) typing has also been developed to increase the discrimination of the *M. tuberculosis* complex species (24, 55). Often a combination of techniques may be used to gain the maximum discrimination between strains (17).

The genome of *M. bovis* has been sequenced (25) and this information has contributed to improved methods of genetic fingerprinting and to the development of PCR assays that define the subspecies of the *M. tuberculosis* complex.

2. Delayed hypersensitivity test

• The tuberculin test (the prescribed test for international trade)

The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold.

Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection. Thus, if a herd/animal is suspected to have been in contact very recently with infected animals, delaying testing should be considered in order to reduce the probability of false-negatives. As the sensitivity of the test is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test. It should be recognised that when used in chronically infected animals with severe pathology, the tuberculin test may be unresponsive. The tuberculin test has not been well validated in most non-bovid and non-cervid species.

The comparative intradermal tuberculin test is used to differentiate between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of exposure to other mycobacteria. This sensitisation can be attributed to the antigenic cross-reactivity among mycobacterial species and related genera. The test involves the intradermal injection of bovine tuberculin and avian tuberculin into different sites, usually on the same side of the neck, and measuring the response 3 days later.

The potency of tuberculins must be estimated by biological methods, based on comparison with standard tuberculins, and potency is expressed in International Units (IU). In several countries, bovine tuberculin is considered to be of acceptable potency if its estimated potency guarantees per bovine dose at least 2000 IU (±25%) in cattle. In cattle with diminished allergic sensitivity, a higher dose of bovine tuberculin is needed, and in national eradication campaigns, doses of up to 5000 IU are recommended. The volume of each injection dose must not exceed 0.2 ml.

• Test procedure

i) A correct injection technique is important. The injection sites must be clipped and cleaned. A fold of skin within each clipped area is measured with callipers and the site marked prior to injection. A short needle, bevel edge outwards and graduated syringe charged with tuberculin attached, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin is then injected. A multi-dose syringe or multiple injection gun may be used provided that delivery of the volume and safety are assured. The dose of tuberculin injected must be no lower than 2000 International Units (IU) of bovine or avian tuberculin. A correct injection is confirmed by palpating a small pea-like swelling at each site of injection. The distance between the two injections should be approximately 12–15 cm. In young animals in which there is no room to separate the sites sufficiently on one side of the neck, one injection must be made on each side of the neck at identical sites in the centre of the middle third of the neck. The skin-fold thickness of each injection site is re-measured 72 hours after injection. The same person should measure the skin before the injection and when the test is read.

ii) A number of alternative methods of interpreting the skin test responses have been adopted, recognising that false-positive reactions may be caused by sensitisation by other mycobacteria and by local inflammation. It is important to recognise that there is a balance between sensitivity and specificity and achieving high concurrent values may not be possible. Appropriate policies need to be in place depending on disease prevalence and according to risk (e.g. where a wildlife reservoir is present). The interpretation is based on observation and the recorded increases in skin-fold thickness. In the single intradermal test (which requires a single injection of bovine tuberculin), the reaction is commonly considered to be negative if only limited swelling is observed, with an increase of no more than 2 mm and without clinical signs, such as diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes. The reaction is considered to be inconclusive if none of these clinical signs is observed and if the increase in skin-fold thickness is more than 2 mm and less than 4 mm. The reaction is considered to be positive if clinical signs, as mentioned above, are observed or if there is an increase of 4 mm or more in skin-fold thickness. Moreover, in *M.-bovis*-infected herds, any palpable or visible swelling should be considered to be positive. Sometimes a more stringent interpretation is used, particularly in a high risk population or in-contact animals. Animals that are inconclusive by the single intradermal test should be subjected to another test after an interval of 42 days to allow desensitisation to wane (in some areas 60 days for cattle and 120 days for deer are used). Animals that are not negative to this second test should be deemed to be positive to the test. Animals that are positive to the single intradermal test may be subjected to a comparative intradermal test or blood test. Any retest should be performed in accordance with the local or national control programmes standard.

iii) In the interpretation of the intradermal comparative test, a reaction is usually considered to be positive if the increase in skin thickness at the bovine site of injection is more than 4 mm greater than the reaction shown at the site of the avian injection. The reaction is considered to be inconclusive if the increase in skin thickness at the bovine site of injection is from 1 to 4 mm greater than the avian reaction. The reaction is considered to be negative if the increase in skin thickness at the bovine site of injection is less than or equal to the increase in the skin reaction at the avian site of injection. This interpretation scheme is used in European Union (EU) countries and is recommended in Council Directive 64/432/EEC (22). Sometimes a more stringent interpretation is used.

iv) In the caudal fold test, a short needle, bevel edge outwards, is inserted obliquely into the deeper layers of the skin on the lateral aspect of the caudal fold, midway along the fold and midway between the hairline and the ventral aspect of the fold. The standard interpretation is that any palpable or visible change is deemed to be a reaction. A modified interpretation is also in use: a positive test is any palpable or visible swelling at the site of the injection that has a caudal fold thickness difference of 4 mm when compared with the thickness of the opposite caudal fold. If an animal has only one caudal fold, it is considered to be test positive if the caudal fold thickness is 8 mm or more.

3. Blood-based laboratory tests

Besides the classical intradermal tuberculin test, a number of blood tests have been used (31). Due to the cost and the more complex nature of laboratory-based assays, they are usually used as ancillary tests to maximise the detection of infected animals (parallel testing), or to confirm or negate the results of an intra-dermal skin test (serial testing). There is also evidence that when an infected animal is skin tested, an enhanced blood test can occur during the following week. This allows for better separation of *in-vitro* blood test responses leading to greater test accuracy. The gamma-interferon assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity.

a) Gamma-interferon assay (the alternative test for international trade)

In this test, the release of a lymphokine gamma interferon (IFN-γ) is measured in a whole-blood culture system. The assay is based on the release of IFN-γ from sensitised lymphocytes during a 16–24-hour incubation period with specific antigen (PPD-tuberculin) (58). The test makes use of the comparison of IFN-γ production following stimulation with avian and bovine PPD. The detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon. It is recommended that the blood samples be transported to the laboratory and the assay set up as soon as practical, but not later than the day after blood collection (9, 50). In some areas, especially where 'nonspecificity' is prevalent, some concerns about the accuracy have been expressed. However, because of the IFN-γ test capability of detecting early infections, the use of both tests in parallel allows detection of a greater number of infected animals before they become a source of infection for other animals as well as a source of contamination of the environment (26). The use of defined mycobacterial antigens such as ESAT 6 and CFP-10 shows promise for improved specificity (7), and these antigens are employed in a number of countries such as the United Kingdom and New Zealand for serial testing. The use of such antigens may also offer the ability to differentiate BCG-vaccinated from unvaccinated animals. In animals that are difficult or dangerous to handle, such as excitable cattle or other bovidae, the advantage of the IFN-γ test over the skin test is that the

animals need be captured only once. The IFN-γ test has been approved for use in a number of national programmes including in the European Union (EU), USA, New Zealand, and Australia. In New Zealand and the United Kingdom for example, the IFN-γ test is used for serial testing (to enhance specificity) and parallel testing (to enhance sensitivity). The test is available as commercial kits for bovine species and primates; however it has been validated in only a few species of these taxa.

b) Lymphocyte proliferation assay

This type of *in-vitro* assay compares the reactivity of peripheral blood lymphocytes to tuberculin PPD (PPD-B) and a PPD from *Mycobacterium avium* (PPD-A). The assay can be performed on whole blood (7) or purified lymphocytes from peripheral blood samples (29). These tests endeavour to increase the specificity of the assay by removing the response of lymphocytes to 'nonspecific' or cross-reactive antigens associated with non-pathogenic species of mycobacteria to which the animal may have been exposed. Results are usually analysed as the value obtained in response to PPD-B minus the value obtained in response to PPD-A. The B–A value must then be above a cut-off point that can be altered in order to maximise either specificity or sensitivity of the diagnosis. The assay has scientific value, but is not used for routine diagnosis because the test is time-consuming and the logistics and laboratory execution are complicated (it requires long incubation times and the use of radio-active nucleotides). As with the IFN-γ test, the lymphocyte proliferation assay should be performed shortly after blood is collected. The test may be useful in wildlife and zoo animals. A blood test comprising lymphocyte transformation assays and ELISA has been reported to have a high sensitivity and specificity in diagnosis of *M. bovis* infection in deer (29). The test is relatively expensive and has not yet been subject to inter-laboratory comparisons.

c) Enzyme-linked immunosorbent assay

There have been numerous unsuccessful attempts to develop clinically useful serodiagnostic tests for tuberculosis. The ELISA appears to be the most suitable of the antibody-detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. It may be helpful in anergic cattle and deer. An advantage of the ELISA is its simplicity, but sensitivity is limited mostly because of the late and irregular development of the humoral immune response in cattle during the course of the disease. Specificity is also poor in cattle when complex antigens such as tuberculin or *M. bovis* culture filtrates are used. However, a comparison of antibody levels to PPD-B and PPD-A has been shown to be useful in increasing specificity in the ELISA (30). The antibody response in deer however seems to develop earlier and more predictably and the sensitivity of a comparative ELISA has been reported to be as high as 85% in this species (30). Improvement may be possible by using a combination of different antigens, including proteins (e.g. MPB70 and MPB83, which are specific but lack sensitivity). Moreover, in *M.-bovis*-infected animals, an anamestic rise has been described, resulting in better ELISA results 2–8 weeks after a routine tuberculin skin test (38). The ELISA may also be useful for detecting *M. bovis* infections in wildlife. In New Zealand, the ELISA is approved as an ancillary parallel test for farmed deer, carried out 13–33 days after the mid-cervical skin test (29). Alternative serum test formats have also been developed. For example, a lateral flow-based rapid test (TB StatPak) has been shown to be useful for detecting tuberculous animals, particularly in some domestic animals, wildlife (37) and zoo animals such as South American camelids , badgers (27), nonhuman primates or elephants (28) where no cellular immunity tests like the gamma-interferon tests are available and where skin testing has been proven unreliable. However, its sensitivity in cattle is relatively low. This test is now licensed in the USA by the USDA for species such as elephants and nonhuman primates and is approved for use in the United Kingdom for badgers.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

At present the only available vaccine against *M. bovis* infections is bacille-Calmette-Guerin (BCG), which is a live attenuated strain of *M. bovis*. This has shown variable efficacy in cattle trials, which may be attributable to various factors including vaccine formulation, route of vaccination, and the degree of exposure to environmental mycobacteria (53). Trials have been conducted on a number of other vaccines, but none has been shown to induce a superior protection to BCG. The efficacy of BCG has been shown to vary in a similar manner to that reported for humans. A number of new candidate vaccines are currently being tested. The genetic make-up of the tuberculosis organism is now being studied in detail and entire genome sequences of *M. tuberculosis, M. bovis* and BCG (Pasteur) have has been published (5, 11, 25). This may be particularly useful in identifying genes associated with virulence and in advancing towards a subunit vaccine. In infected countries where there is no test and slaughter control scheme, BCG vaccination may be used to reduce the spread of infection in cattle; however, there is no solid knowledge of long-term reduction in prevalence and safety for human beings and the environment. Before embarking on a vaccination programme, the vaccination schedule must be optimised for local conditions. Typical dosage would be from 10^4 to 10^6 colony-forming units given subcutaneously. Vaccine should be based on the standard reference strain, BCG Pasteur or Danish (60). It is important to recognise that use of vaccine will compromise tuberculin skin tests or other immunological tests relying on the use of tuberculin

as diagnostic antigen. Cattle vaccination should not therefore be used in countries where control or trade measures based on such testing are in operation. However, significant progress has been made in the development of so-called DIVA antigens that allow the differentiation of BCG vaccinated from *M. bovis* infected animals, particularly when used in the gamma-interferon test (6, 10, 52, 56). Such antigens are based on the use of antigens that are encoded on *M. bovis* gene regions that are deleted in BCG (such as ESAT-6 and CFP-10 [6, 56]), that are under-expressed in some BCG strains (such as MPB83), or are not secreted by BCG (such as Rv3615c [52]). It can therefore be envisaged that BCG vaccination could be applied in combination with such DIVA tests once these reagents have been fully validated and the legal framework amended accordingly. BCG vaccines may also be used to reduce spread of *M. bovis* in wildlife reservoirs of infection. Prior to using the vaccine, it is essential to validate the delivery system for the particular wildlife species. The environmental impact of the vaccine on other wildlife species must also be considered.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Tuberculin preparations were originally made from the heat-treated products of growth and lysis of *M. tuberculosis* or *M. bovis* (known as human and bovine tuberculins, respectively) grown in glycerol broth. In the 1940s, the 'heat-concentrated synthetic medium tuberculins' or HCSM tuberculins, prepared from cultures in a synthetic liquid medium, replaced the 'old' tuberculins. The old and HSCM tuberculins have been replaced, almost world-wide, with the purified protein derivatives or PPDs. Bovine PPDs prepared with the *M. bovis* production strain AN5 are more specific for detecting bovine tuberculosis than human PPDs prepared with *M. tuberculosis.*

• Production of tuberculin

1. Seed management

a) Characteristics of the seed

Strains of *M. bovis* used to prepare seed cultures must be identified to species level by appropriate tests. A record must be kept of their origins and subsequent history. Seed cultures must not be passaged more than five times. The production strains *M. bovis* AN5 or Vallee are the most commonly used.

b) Method of culture

If the source culture was grown on solid medium, it is necessary to adapt the organism to grow as a floating culture (e.g. by incorporating a sterile piece of potato in the culture flasks of liquid media, such as Watson Reid's medium). When the culture has been adapted to liquid medium, it may be used to produce the master seed lot, which is preserved in freeze-dried form. This is used to inoculate media for the production of the secondary seed lots, which must not be more than four culture passages from the master seed. The secondary seed is used to inoculate production cultures (1, 32).

The production culture substrate must be shown to be capable of producing a product that conforms to recognised international standards (World Health Organization [WHO], European Pharmacopoeia or other recognised control authorities). It must be free from ingredients known to cause toxic or allergic reactions.

c) Validation

The strains of *M. bovis* used as seed cultures must be shown to be free from contaminating organisms.

Seed lots must be shown to be efficacious in producing tuberculin with sufficient potency. The necessary tests are described in Section C.4 below.

2. Method of manufacture

The organism is cultured in a synthetic medium, the protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), then washed and resuspended. PPD tuberculin is recommended as it can be standardised more precisely.

An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. Mercurial derivatives should not be used. The product is also dispensed aseptically into sterile, neutral glass containers, which are sealed so as to preclude contamination. The product may be freeze-dried.

3. In-process control

The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving.

As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask.

In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be 6.6–6.7.

The protein level of the PPD concentrate is determined by the Kjeldahl or other suitable method. Total nitrogen and TCA precipitable nitrogen are usually compared.

The final product should be bioassayed in guinea-pigs. Potency and specificity assays are carried out in comparison with a reference tuberculin (PPD). Further dilutions are made with a buffer according to the protein content and the required final concentration, usually 1.0 mg/ml (1, 32).

4. Batch control

Samples should comply with the officially recognised standards for the production of tuberculin as set out in the European Pharmacopoeia or equivalent regulatory standards.

a) Sterility

Sterility testing is generally performed according to international guidelines (see also Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally into at least two guinea-pigs, dividing the dose between them. It is desirable to take a larger sample, such as 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and are then examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

c) Sensitising effect

To test the sensitising effect, three guinea-pigs that have not been treated previously with any material that could interfere with the test are injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

d) Potency

Potency is determined by comparison with a reference preparation of bovine tuberculin in guinea-pigs sensitised with *M. bovis*.

As early as the 1960s, the European Economic Community (EEC, now the EU) recognised an EEC standard for bovine PPD, which was given a potency of 50,000 provisional Community tuberculin units per mg of PPD, and was dispensed in the lyophilised state. Unfortunately, the number of freeze-dried ampoules was not sufficient for the WHO's requirements and therefore it was decided to produce a new bovine PPD preparation that could be designated by the WHO as the new international standard for bovine PPD tuberculins.

This new bovine PPD standard had to be calibrated against the existing EEC standard. Based on international collaborative assays, both in guinea-pigs and cattle, it was found that the new bovine standard had a relative potency of 65% against the EEC standard. Therefore, in 1986, the WHO officially gave the international standard for bovine PPD tuberculins a unitage of 32,500 IU/mg. This means that the provisional

Community tuberculin units are equipotent with the IUs. The European Pharmacopoeia has also recognised the WHO international standard for bovine PPD.

In order to save the stock of the actual international standard, it is desirable that the countries where bovine PPD tuberculin is produced, establish their own national reference preparations for bovine PPD as working standards. These national reference preparations must have been calibrated against the official international standard for bovine PPD, both in guinea-pigs and cattle (39, 51, 59).

- **Standardisation in guinea-pigs**

The guinea-pigs are sensitised with a low dose (e.g. 0.001 or 0.0001 mg wet weight) of live bacilli of a virulent strain of *M. bovis* 5–7 weeks prior to the assay. The bacilli are suspended in physiological saline, and a deep intramuscular injection of 1 ml is made on the medial side of the thigh. At the time of the assay, the guinea-pigs infected with the low dose of *M. bovis* should still be in good health and the results of numerous post-mortem examinations carried out shortly after the standardisation assays should show that the guinea-pigs do not suffer from open tuberculosis and thus are not excreting tubercle bacilli.

An alternative potency test can be used that does not use live pathogenic mycobacteria and is more suitable for laboratories that do not have isolation areas for safe housing of infected guinea-pigs. In addition, this option is more convenient in terms of experimental animal welfare. This tuberculin potency test is performed as follows: the PPD tuberculin is bioassayed in homologously sensitised guinea-pigs against the standard for bovine PPD tuberculin by an eight-point assay comprising four dilutions corresponding to about 20, 10, 5 or 2.5 IU. The injection volume is 0.1 ml. In this assay, two test tuberculins are compared with standard tuberculin in eight guinea-pigs, applying eight intradermal injections per animal and employing a Latin square design. The guinea-pigs are sensitised with inactivated bacilli of *M. bovis,* 5–7 weeks before the assay. The bacilli are suspended in buffer and made into an emulsion with Freund's incomplete adjuvant. A deep intramuscular injection is made on the medial side of the thigh, using a dose of 0.5 ml.

A suitable assay for potency is as follows: The produced PPD tuberculins are bioassayed in homologously sensitised guinea-pigs against the standard for bovine PPD tuberculin by a six-point assay comprising three dilutions at five-fold intervals of each tuberculin. The dilutions of the tuberculin preparations are made in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Volumes of 0.001, 0.0002 and 0.00004 mg tuberculoprotein corresponding to the international standard for PPD of 32, 6.4 and 1.28 IU, respectively, are chosen because these amounts give good readable skin reactions with acceptable limits. The injection volume is 0.2 ml. In one assay, two test tuberculins are compared with the standard tuberculin in nine guinea-pigs, applying eight intradermal injections per animal and employing a balanced incomplete Latin square design (23).

Normally, the reading of the assays is done 24 hours after the injection of the tuberculins, but a second additional reading can be performed after 48 hours. The different diameters of erythema are measured with callipers in millimetres and recorded on assay sheets. The results are statistically evaluated using standard statistical methods for parallel-line assays according to Finney (23). The relative potencies of the two test tuberculins are calculated with their 95% confidence limits, the slopes of the log dose–response curves for each preparation (increase in mean reaction per unit increase in log dose) and the F ratios for deviations from parallelism.

According to the European Pharmacopoeia, the estimated potency for bovine tuberculins must be not less than 66% and not more than 150% of the potency stated on the label.

- **Standardisation of bovine tuberculin in cattle**

According to WHO Technical Report Series No. 384, potency testing should be performed in the animal species and under the conditions in which the tuberculins will be used in practice (59). This means that bovine tuberculins should be assayed in naturally infected tuberculous cattle. As this requirement is difficult to accomplish, routine potency testing is conducted in guinea-pigs. However, periodic testing in tuberculous cattle is necessary and standard preparations always require calibration in cattle. The frequency of testing in cattle can be reduced if it is certain that the standard preparations are representative of the routine issue tuberculins and that the production procedures guarantee consistency.

A suitable potency assay for bovine tuberculins in cattle is as follows: The test tuberculins are assayed against a standard for bovine PPD tuberculin by a four-point assay using two dilutions at five-fold intervals of each tuberculin. For the standard, 0.1 and 0.02 mg of tuberculoprotein are injected as these volumes correspond with about 3250 and 650 IU if the international standard for bovine PPD turberculin is used. The test tuberculins are diluted in such a way that the same weights of protein are applied. The injection volume is 0.1 ml, and the distance between the middle cervical area injection sites is 15–20 cm. In one assay, three test tuberculins are compared with the standard tuberculin in eight tuberculous cattle, applying eight intradermal injections per animal in both sides of the neck, and employing a balanced complete Latin square

design. The thickness of the skin at the site of each injection is measured with callipers in tenths of a millimetre, as accurately as possible before and 72 hours after injection (33).

The results are statistically evaluated using the same standard methods for parallel-line assays as employed in the potency tests in guinea-pigs.

e) Specificity

A suitable assay for specificity is as follows: three bovine test tuberculins are assayed against the standard for avian PPD tuberculin (or three avian test tuberculins against the standard for bovine PPD tuberculin) by a four-point assay in heterologously sensitised guinea-pigs, comprising two dilutions at 25-fold intervals of each tuberculin. Quantities of 0.03 mg and 0.0012 mg of test tuberculoprotein, corresponding to approximately 1500 and 60 IU, are chosen because these doses give good readable skin reactions. The injection doses of the standard are lower, namely 0.001 mg and 0.0004 mg. In one assay, three test tuberculins are compared with the standard tuberculin in eight guinea-pigs by applying eight intradermal injections per animal and employing a balanced complete Latin square design. The reading of the results and the statistical evaluation are identical with the potency test.

f) Stability

Provided the tuberculins comply with the legislative standards required for production and are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the expiry date as specified in the licence for production of tuberculin. For long-term storage, it is recommended to keep the PPD in a concentrated form rather than the diluted form and the concentrate should also be stored in the dark.

g) pH control

The pH should be between pH 6.5 and 7.5.

h) Protein content

The protein content is determined as indicated in Section C.3 In-process control.

i) Storage

During storage, liquid bovine tuberculin should be protected from light and held at a temperature of 5±3°C. Freezing of the liquid product may compromise the quality. However, freeze-dried preparations can be prepared and they may be stored at higher temperatures (but not exceeding 25°C); they should be and protected from light. Periods of exposure to higher temperatures or to direct sunlight should be kept to a minimum.

j) Preservatives

Antimicrobial preservatives or other substances that may be added to a tuberculin must have been shown not to impair the safety and effectiveness of the product.

The maximum permitted concentration for phenol is 0.5% (w/v), and for glycerol it is 10% (v/v).

k) Precautions (hazards)

Experience both in humans and animals led to the observation that appropriately diluted tuberculin, injected intradermally, results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive individuals, severe, generalised reactions are extremely rare and limited. But experience has shown that a hypersensitive operator can acquire severe generalised signs after accidental intradermal contact (needle stab-wound) with bovine tuberculin. These individuals should be advised not to carry out the tuberculin skin test with the high dose of 2000–5000 IU tuberculin, which is about 1000 times the normal human dose of 5 IU.

5. Tests on the final product

a) Safety

A test for the absence of toxic or irritant properties must be carried out (see Section C.4.b).

b) Potency

The potency of tuberculins must be estimated by biological methods. These methods must be used for HCSM and PPD tuberculins; they are based on comparison of the tuberculins to be tested with a standard reference preparation of tuberculin of the same type (see also Section C.4.d).

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NB: There are OIE Reference Laboratories for Bovine tuberculosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).