CHAPTER 3.1.13.

MAMMALIAN TUBERCULOSIS (INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS* COMPLEX)

SUMMARY

Mammalian tuberculosis is a chronic bacterial disease of animals and humans caused by members of the Mycobacterium tuberculosis *complex* (*MTBC*). *Within the complex, important variants include* M. bovis, M. caprae *and* M. tuberculosis. *The disease is global in distribution, with only a few countries considered free. It is a major infectious disease of cattle, other domesticated animals, and certain wildlife populations. Zoonotic tuberculosis resulting from transmission to humans constitutes a public health problem.*

Aerosol exposure to the agent is the most frequent route of infection, but infection by ingestion of contaminated material also occurs. Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Tuberculosis is typically a chronic disease, with clinical signs that may appear after several months to years. 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. Tuberculosis in cattle, goats and deer is usually diagnosed in the live animal by tests for cellular immunity (*skin test or gamma interferon test*). *Enzyme-linked immunosorbent assay* (*ELISA*) *and lateral flow tests for serum antibodies can be useful for wildlife studies. After death, infection is diagnosed by necropsy, histopathological, bacteriological and nucleic acid detection techniques.*

Detection of the agent: *Bacteriological examinations may consist of the demonstration of acid-fast bacilli by microscopic examination of tissues or secretions, which provides presumptive identification. Confirmatory tests include the isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA techniques. The polymerase chain reaction* (*PCR*) *test may be used for the detection of members of the MTBC in clinical specimens. Spacer oligonucleotide typing* (*spoligotyping*), *mycobacterial interspersed repeat units–variable number tandem repeat* (*MIRU-VNTR*) *and whole genome sequencing are used for genotyping members of the MTBC for purposes of strain tracking and epidemiology.*

Delayed hypersensitivity test **(*tuberculin skin test*):** *This test is the standard method for detection of tuberculosis in live cattle, small ruminants, deer, pigs and camelids, among other species. The single test involves measuring the skin response after intradermal injection of tuberculin* (*purified protein derivative – PPD*). *The comparative tuberculin skin test with bovine* (*PPD-B*) *and avian* (*PPD-A*) *tuberculin is used mainly to differentiate between animals infected with the* M. tuberculosis *complex 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 other sensitising organisms. The recommended dose of bovine PPD-B 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. Defined antigen skin tests are being evaluated and should prove useful for differentiation of infected from vaccinated animals.*

Blood-based laboratory tests: *Diagnostic blood tests include the interferon-gamma release assay* (*IGRA*), *which measures cellular immune responses to tuberculin or defined antigens, and the indirect ELISA and lateral flow assays, which detect antibody responses. The logistics and laboratory execution of some of these assays 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 validation data for some species.*

Requirements for vaccines and diagnostic biologicals: *The only currently available vaccine against* M. bovis *infections is bacille-Calmette-Guerin (BCG). However, BCG may sensitise animals to the tuberculin skin test and other tuberculin-based immunological tests. For this reason, vaccination of cattle is prohibited in many countries. Defined antigen skin tests or IGRA, once validated, should enable differentiation of infected from vaccinated animals. BCG vaccines have been granted regulatory approval for the immunisation of certain wildlife species such as the badger, and trials are in progress to evaluate the use of BCG in cattle in a number of countries.*

Methods for the production of bovine PPD tuberculins should comply with standard requirements for source materials, production procedures 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 International Units (IUs) calibrated against the International Standard Bovine Tuberculin (ISBT).

A. INTRODUCTION

Mammalian tuberculosis is a chronic granulomatous disease of animals and humans that results from infection with pathogenic members of the *Mycobacterium tuberculosis* complex (MTBC). MTBC members belong to the family Mycobacteriaceae and are Gram-positive, acid-fast bacilli. The taxonomy of organisms in the MTBC is in flux. Recent genomic analyses suggest that all MTBC members belong to a single species – *M. tuberculosis*, with *M. africanum*, *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* considered heterotypic synonyms (variants) of *M. tuberculosis*; and *M. canettii*, *M. mungi*, and *M. orygis* recognised as strains of *M tuberculosis*. To retain linkage with historical nomenclature, the more widely recognised designations are used in this Chapter instead of infra-subspecific designations. Hence, we continue to use designations *M. bovis* or *M. caprae* instead of *M. tuberculosis* var. bovis or *M. tuberculosis* var. caprae, respectively, for ease of prior association.

While there is considerable evidence of host-association amongst members of the MTBC, currently all mammalian species are considered to be susceptible to tuberculosis, and nearly identical disease may potentially result in any host species from infection with any member of the complex. The primary pathogens associated with domesticated and wild animals include *M. bovis*, *M. caprae*, *M. microti, M. orygis* and *M. pinnipedii.* Human exposure to any member of the MTBC (apart from BCG vaccine strain) may result in zoonotic infection. *Mycobacterium tuberculosis* and *M. africanum* are primarily pathogens of humans, but these too are known to infect animals. While *M. canettii* has only been described in humans in Africa, it is considered to be a spillover transmission from a potential wild animal reservoir that has yet to be determined. *Mycobacterium mungi* has been recovered from banded mongooses (*Mungos mungo*), and *M. suricattae* from meerkats (*Suricata suricatta*), but neither organism has yet been described from other host species. *Mycobacterium microti* is often diagnosed in cats due to transmission from the rodent maintenance hosts. *Mycobacterium microti* has also been found widespread in wild boar. *Mycobacterium tuberculosis sensu stricto* may be found as anthroponoses in elephants and companion animals. Within the complex, *M. bovis*, *M. caprae* and *M. tuberculosis* are considered important variants in terms of broader host range, and public health perspective.

The disease is global in distribution. For up-to-date information, consult the WOAH WAHIS interface[1]. A number of countries are considered free of tuberculosis in livestock. In others, with active animal tuberculosis surveillance and control programmes, there are only infrequent disease reports. The disease remains endemic and largely uncontrolled in Africa, Asia, Latin America and most countries in the Middle East. Wildlife reservoirs of tuberculosis have been described in several countries, and members of the MTBC are frequently recovered from free-living and captive wildlife hosts. Spillover from suspected maintenance wildlife reservoir hosts to cattle and other livestock have been reported in certain regions of Canada (from elk, *Cervus canadensis*), the Iberian peninsula (wild boar, *Sus scrofa,* and several cervid species), Ireland and the United Kingdom (European badger, *Meles meles*), New Zealand (primarily the brush-tailed possum, *Trichosurus vulpecula*), South Africa (African buffalo, *Syncerus caffer*), and in certain regions of the United States (white-tailed deer, *Odocoileus virginianus*). In regions where MTBC circulates in wildlife, multiple spillover hosts ranging from rodents and small mammals to carnivores and ungulates may exist. In regions with high prevalence of human tuberculosis and close interface with livestock, humans may represent a maintenance host for livestock infections, with spillover from humans to animals reported for *M. tuberculosis, M. bovis* and *M. orygis*.

There is increasing evidence of regional and geographical variation in distribution of members of the MTBC reported amongst livestock species. For instance, while *M. bovis* appears the predominant cause of tuberculosis in

1 https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

cattle in Africa, much of Western Europe, and the Americas, *M. caprae* appears to be the major cause of tuberculosis in cattle in central European countries. Similarly, there is increasing evidence of *M. tuberculosis*, *M. orygis*, and *M. caprae* representing major causes of TB in cattle in South Asia.

Tuberculosis is typically a chronic disease, with clinical signs that may appear after several months to years. Infected animals may remain quiescent for an extended duration and reactivate after several years or when immunosuppressed. Acute or per-acute infections occur rarely in animals, and if they do, are likely in animals with an underlying co-morbidity such as being immunosuppressed.

Infection typically occurs through the inhalation, ingestion or direct contact of infected material through mucus membranes or breaks in the skin. Risk of infection may depend upon dose, route of infection, or a number of pathogen, host or animal husbandry related variables. Younger animals are considered to be more susceptible to infection, as are immunosuppressed, pregnant or malnourished animals.

While inhalation of infected respiratory droplets is considered as the primary mode of transmission for MTBC in livestock species, infection through ingestion of contaminated milk (particularly in neonates), feed, or water as well as through contact with infected exudates, urine, faeces, as well as vaginal secretions and semen may also occur. Inhaled bacilli are phagocytosed by alveolar macrophages. Depending on factors including dose and route of infection along with pathogen and host covariates, the pathogen may not survive to initiate an active infection, or the macrophages may successfully clear infection. When the infection is not immediately cleared, the organism may remain quiescent, or actively proliferate. Active proliferation of the organism and the failed host response to contain the infection results in focal or disseminated lesions that consist of dead and degenerate macrophages surrounded by multiple lymphoid origin cells. These later coalesce into multinucleated giant cells, with caseation or calcification of the necrotic centre followed by formation of a granuloma surrounded by a fibrous capsule – the prototypical “tubercle”.

These granulomatous lesions vary considerably in size and are often encapsulated. They may be caseous, caseo-calcareous, or calcified in cattle and many other mammalian species. In cervids or other animals with a more rapidly progressing disease, pus-filled abscesses are apparent instead of the classic tubercles. While almost any organ system might appear lesioned, in animals where inhalation is the route of infection, tubercles are often observed in the lungs as well as in cranio-thoracic lymph nodes. In contrast, in animals in which the primary route of infection is ingestion, abdominal organs as well as mesenteric lymph nodes are more likely to harbour lesions. In some animals, including those with advanced disease, widely disseminated infections may occur and result in miliary tuberculosis with small foci scattered throughout a tissue.

The involvement of draining regional lymph nodes is common in cases of active TB. In a subset of animals, lesions remain localised, resulting in chronic disease and persistent infection. In some immunosuppressed animals or those with advanced infection, lymphatic or haematogenous dissemination may occur. This results in a generalised infection, or miliary TB, with nodular lesions in multiple organ systems including the lungs, liver, kidney, spleen, mammary glands, gastrointestinal tract and the central nervous system. In such advanced cases, the disease is invariably fatal if left untreated.

There are no pathognomonic or distinctive clinical features associated with mammalian tuberculosis. The disease has a typical slow onset, and infected animals may actively transmit infection without displaying any apparent signs. The most frequently observed clinical signs in cattle include a progressive weight loss, weakness, inappetence, low-grade elevated or fluctuating body temperature, coughing and lymphadenopathy.

The enlargement of superficial lymph nodes is often observed. In advanced cases, these may rupture and drain. Retropharyngeal lymph nodes may also be affected, and the enlargement of deeper lymph nodes may result in obstruction of blood vessels or lymphatics in the respiratory and or gastrointestinal tracts. Respiratory involvement is usually indicated by a moist, intermittent cough that is worsened in cold conditions or during exertion, with the onset of dyspnoea or tachypnoea in more advanced cases. Alterations in gastrointestinal function and motility, including diarrhoea or, less frequently, constipation may be observed. Red deer, other cervids and camelids often have an accelerated disease (as compared with cattle) with multi-organ involvement. Camelids and equids may present with more gastrointestinal signs. Elephant do not show typical signs until very late in disease.

The ante-mortem diagnosis of tuberculosis in animals may be performed by direct detection of the organism, or indirectly, by evidence of host immune response to infection using the tuberculin skin test (TST), the interferon-gamma release assay (IGRA) or antibody assays. In some animal species such as non-human primates and small companion animals, radiological examination and echography may be used to supplement other tests. Given the chronic nature of infection and absence of definitive clinical signs, evidence of tuberculosis in animals is often only identified post-mortem during slaughter surveillance or at necropsy by identification of characteristic lesions. Since

a substantial fraction of animals that are classified as reactors by the tuberculin skin test may not show visible lesions at necropsy, bronchial, mediastinal and other lymph nodes are routinely sampled during slaughter surveillance, for the confirmatory detection of infection.

Direct microbiological detection of tuberculosis is most often performed by culture of tissue samples, exudates and other suspect fluids or secretions, or trunk washes in elephants. Presumptive diagnoses may be made by microscopic identification of characteristic acid-fast bacilli in secretions or tissue samples or histopathological staining of multinucleated giant cells with central caseating necrosis; however, the microscopic test can often give negative results, even in sick animals, due to the limited presence of mycobacteria. Mycobacterial culture is performed by growth on selective solid or liquid media, followed by confirmatory assays that include identification of mycobacterial specific nucleic acid or other microbial biomarkers. Direct detection approaches such as culture are the definitive techniques for diagnosis of tuberculosis in animals, but are time-consuming and difficult to perform. For this reason rapid methods such as polymerase chain reaction (PCR) are increasingly used for the direct detection of DNA from biological specimens.

Evidence of immune response of animals to MTBC organisms is the most commonly used indirect approach for the identification of presumptively infected animals. The primary screening test in animals, the TST, is an *in vivo* measure of the delayed hypersensitivity response to antigens present in MTBC organisms. The assay is carried out by the intradermal injection of a purified protein derivative (or tuberculin) derived from a well-defined strain of *M. bovis* (PPD-B), and measurement of the subsequent increase in skin-fold thickness at the injection site. The site of test administration depends on species of animal. In regions with high exposure of animals to environmental mycobacteria, the difference in skin thickness to PPD-B and purified protein derivative from a *M. avium* strain (PPD-A) is used to improve specificity, but often at the cost of sensitivity. Given the historical use and success in enabling test and remove based control programmes, the TST is the preferred test for diagnosis of tuberculosis and for screening pre-trade.

The *in vitro* IGRA provides an alternative measure of the cellular immune response to infection. This assay measures the release of gamma interferon from blood cells stimulated with antigens derived from MTBC organisms. This assay is often used as an ancillary test to remove additional positive animals and may be more convenient than the skin test since it does not require repeated handling of the animal or extended intervals prior to retesting. However, since the IGRA requires time-bound stimulation of live blood cells, it may prove costly, cumbersome or difficult to implement, especially in remote or low resource settings.

Since specific antibodies to MTBC infection are thought to develop later during infection, serological or antibody tests are considered less sensitive than the cell mediated immune response assays. The most frequently used serological assays include enzyme linked immunosorbent assays (ELISA) or lateral flow assays that measure antibodies to specific MTBC antigens. Antibody assays may be particularly useful in detection of evidence of immune response to tuberculosis in wildlife species. In livestock as well as certain cervid and camelid species, the prior administration of the skin test antigen within a specified time period is needed to ensure assay sensitivity.

Vaccines such as bacille-Calmette-Guerin (BCG) have been evaluated for use in livestock and wildlife species. In cattle, BCG is reported to have only modest levels of direct efficacy based on meta-analyses of experimental challenge studies and field trials. However, taken together with potential indirect effects of reduced onward transmission, simulation models suggest that implementation of vaccine programmes in endemic regions where test and cull are not feasible, may considerably accelerate TB control efforts.

B. DIAGNOSTIC TECHNIQUES

Most members of the MTBC are zoonotic organisms. Animal specimens and bacterial cultures that may contain live mycobacteria should be handled at an appropriate biosafety and containment level determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). This should include use of biological safety cabinets where appropriate.

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed. For this reason, its diagnosis in individual animals and establishment of surveillance and eradication programmes were not possible prior to the development of tuberculin by Koch in 1890. Intradermal inoculation of tuberculin, a concentrated sterile culture filtrate of tubercle bacilli, provides an indirect means of detecting previous exposure or active infection by measuring the delayed hypersensitivity immune response. In companion animals and in valuable exotic/zoo species, the application of radiography is frequently applied as a presumptive diagnostic.

The presence of species of the MTBC 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 sterile. Non-sterile sampling containers may result in the failure to identify members of the MTBC due to the rapid growth of contaminating environmental mycobacteria or other organisms. 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 as summarised in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*. Delivery of specimens to the laboratory within 48 hours greatly enhances the chances of cultural recovery of MTBC members. 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, or when refrigeration is not possible, the tissue may be stored in a saturated solution of sodium borate, but only for limited periods, no longer than 30 days.

The main diagnostic methods and their fitness for purpose are summarised in Table 1 and described in the following sections. This is not an exhaustive listing but indicates test appropriate for different purposes in different host species.

Table 1. Test methods available for use in cattle, goats, and camelids and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Acid-fast staining and microscopy	–	–	–	+	–	–
Bacterial isolation	++	–	++	+++	++	–
Histopathology and antigen detection	+	–	+	+	–	–
Real-time PCR (direct from specimens)	++	–	++	+++	++	–
Detection of immune response						
Delayed hypersensitivity skin test	+++ (**+++** / **+**)	+++ (**++** / **+**)	+++ (**++** / **+**)	++	+++ (**++** / **+**)	–
IGRA	++ (**++** / **+**)	++ (**+** / **+**)	+++ (**++** / **+**)	+ (**–** / **–**)	+++ (**++** / **+**)	–
ELISA antibody test	+ (**–** / **++**)	+ (**+** / **++**)	+ (**–** / **++**)	–	+ (**–** / **++**)	–
Lateral flow antibody test	+	+	+	–	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose. Methods whose fitness for purpose differ between cattle, goats and camelids are represented in black colour for cattle, and differences for **goats** and **camelids** shown in parentheses in red and blue colours, respectively.
PCR = polymerase chain reaction; IGRA = Interferon gamma release assay; ELISA = enzyme-linked immunosorbent assay.

1. Detection and identification of the agent

1.1. Microscopic examination

Mycobacterium spp. may be suspected microscopically on direct smears from clinical samples and on prepared tissue materials using the classic acid-fast 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 with haematoxylin and eosin staining if the tissue has characteristic histological lesions representing the animal's immune response to the infection (caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, acid-fast organisms may not be detected in histological sections, even though MTBC organisms 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).

1.2.Culture

Successful recovery of mycobacteria by bacteriological culture depends on the sampling technique at the abattoir, the type of tissue specimens collected and their preservation in transit to the laboratory (refrigeration or freezing), together with the chemical decontamination step and the type of culture media chosen to grow mycobacteria.

The best tissues for the isolation of MTBC organisms are mainly collected during post-mortem examination from animals tested positive to tuberculin or interferon gamma tests. Abnormal lymph nodes and parenchymatous organs with TB-compatible lesions should be collected for examination and culture. If pathological lesions are not detected then, specific lymph nodes from the head, respiratory and/or digestive locations should be taken for the same purpose. Pets or zoo animals with suspected tuberculosis can be tested with biopsy specimens from lymph nodes, oral swabs or tracheo-bronchial wash.

To process specimens for culture, the tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, pooled, and homogenised using a stomacher or tissue grinder (e.g. mortar, pestle, blender, etc.) with sterile PBS, followed by a mandatory decontamination. The purpose of the decontamination step is to avoid growth of other bacteria present in the tissue samples. Due to the cell wall characteristic of mycobacteria, they can resist certain detergents or extreme changes in the pH. A variety of protocols has been described, for example treatment with 0.375–0.75% hexadecylpyridinium-chloride detergent (HPC), 2–4% sodium hydroxide, 5% oxalic acid or 4% sulfuric acid, among others. It is important to control the time of exposure of tissues to the decontaminant to avoid death of the mycobacteria. Depending on the amount of tissue and decontaminant it usually ranges between 10 and 20 minutes. Where acid or alkali methods are used they must then be restored to a neutral pH after decontamination at room temperature. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination.

For primary isolation, the sediment is usually inoculated on to a set of solid and/or liquid media. Different solid egg-based media can be used such as Lowenstein–Jensen, Coletsos base or Stonebrink. These media should contain either pyruvate or pyruvate and glycerol. Solid agar-based media such as Middlebrook 7H10 or 7H11 or blood-based agar medium may also be used (Cousins *et al*., 1989, Gormley *et al.*, 2014).

Liquid culture systems are used routinely in some laboratories; in these systems growth is measured by fluorometric means. Liquid media offer a shorter time of growth and relatively rapid mycobacterial detection. However, their disadvantages are a higher contamination rate and the impossibility of making an initial diagnosis based on the colonies' morphological characteristics. PCR is a useful method to confirm bacterial growth in liquid media. Additionally, it should be considered that some decontaminants (e.g. HPC) are not compatible with some liquid systems.

Cultures are incubated for a minimum of 6 weeks, preferably for 10–12 weeks (and up to 16 weeks when there is a suspicion of *M. microti*) at 37°C with or without CO_2. The incubation period also depends on the use of liquid or solid media. The media should be in tightly closed tubes to avoid desiccation. When solid media are used slopes are examined for macroscopic growth at weekly intervals during the incubation period. When growth is visible, smears can be prepared and stained by the Ziehl–Neelsen technique.

Alternatively, DNA can be extracted from colonies and any specific PCR for mycobacteria or MTBC species can be performed (see section 1.3 Nucleic acid recognition methods). Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used, the presence of tuberculosis compatible lesions and/or the initial bacterium load in the tissue specimen.

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

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis; however every isolate needs to be confirmed. It is often necessary to distinguish *M. bovis* from the other members of the MTBC, including *M. tuberculosis, M. africanum M. caprae. M. microti* and *M. pinnipedii*.

Mycobacterium avium or other environmental mycobacteria may also be isolated from tuberculosis-like lesions in cattle. In such cases, definitive identification of the causative agent may be needed, and mixed infections excluded.

Isolates may be presumptively identified from their cultural and biochemical properties, although this can be time consuming due to the slow growth of the members of the MTBC. 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. One approach to differentiate MTBC from non-tuberculous mycobacteria is through growth in media containing 500 mg/litre p-nitrobenzoic acid. MTBC do not grow while most non-tuberculous mycobacteria do.

Mycobacterium bovis is a microaerophillic and nonchromogenic bacterium sensitive to thiophen-2-carboxylic acid hydrazide (TCH) and to isonicotinic acid hydrazide (INH). Drug susceptibility testing can be evaluated by different protocols, including growth on solid 7H10/7H11 Middlebrook agar medium, on egg-containing media, or liquid systems (ECDC, 2018). 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.

1.3. Nucleic acid recognition methods

1.3.1. Polymerase chain reaction

Rapid identification of isolates to the level of MTBC can be performed by use of polymerase chain reaction (PCR) targeting 16S–23S rRNA, insertion sequences such as IS*6110* or IS*1081*, or similar sequences representing MTBC-specific targets. Species-level identification of members of the MTBC may be achieved with molecular genetic approaches for detecting the presence or absence of genomic "regions of differences" or species-defining (single-nucleotide) polymorphisms. Sub-species identification may be achieved with molecular typing approaches such as spoligotyping or, increasingly, whole genome sequence analyses to aid in strain differentiation as well as in molecular epidemiologic analyses.

PCR is routinely used for the detection of members of the MTBC in clinical specimens (mainly sputum) in humans, and is increasingly applied for the diagnosis of tuberculosis in animals. A number of commercially available assays and various 'in-house' methods have been evaluated for the detection of members of the MTBC in fresh samples or fixed tissues. Of key importance is that the DNA extraction method to be employed includes a mechanical lysis step as well as the chemical lysis (Lorente-Leal *et al*., 2019). Various MTBC- or species-specific targets for amplification have been identified as noted above. The most common targets for PCR identification of members of the MTBC are the multicopy insertion elements IS*6110* or IS*1081* (or both). Several assays for real-time PCR-based detection of these two targets have been reported, with good to excellent performance characteristics as compared with either culture or histopathology (e.g. Courcoul *et al.,* 2014; Sanchez-Carvajal *et al.,* 2021). Examples of well validated primer and probe sequences include those in use for the USDA national animal tuberculosis surveillance program that target IS*1081*:

IS1081_F1 5'-GGC-TGC-TCT-CGA-CGT-TCA-TC;
IS1081_RI 5'-CGC-TGA-TTG-GAC-CGC-TCA-T;
IS1081_PI 5'-CTG-AAG-CCG-ACG-CCC-TGT-GC;

and for IS*6110*:

IS6110_F1 5'-CAG-GAC-CAC-GAT-CGC-TGA-TC;
IS6110_R1 5'- CTG-CCC-AGG-TCG-ACA-CAT-AG;
IS6110_P1 5'-CGT-CCC-GCC-GAT-CTC-GTC-CA,
amongst others (Dykema *et al.,* 2016).

Amplification products are preferably detected by hybridisation with probes in real-time assays or visualised by gel electrophoresis and staining. Commercial kits and the 'in-house' methods, in fresh, frozen or boric acid-preserved tissues often do not perform well during interlaboratory comparisons, and hence may require additional validation to ensure accuracy. False-positives, or false-negative results in specimens containing low numbers of bacilli, are often of concern. Pre-analytical sample processing including decontamination and DNA extraction procedures, elimination of polymerase enzyme inhibitors, etc., have considerable impact on assay performance. Use of standard operating procedures, internal and external extraction and amplification controls, procedures for the prevention of cross-contamination, and application of closed tube real-time PCR assays are highly recommended for the reliable molecular genetic identification of members of the MTBC.

1.3.2. DNA fingerprinting

A variety of DNA-fingerprinting techniques has been developed to distinguish members of the MTBC for molecular epidemiological purposes (Guimaraes & Zimpel, 2020; Merker *et al.,* 2017). These methods are useful in identifying and tracing sources of origin, as well as for tracking transmission and spread of MTBC members within herds and globally. Spacer oligonucleotide typing, or spoligotyping, is a PCR-based method that has been widely used for genotyping members of the MTBC (Kamerbeek *et al.,* 1997). The method involves amplification of chromosomally-encoded loci that contain a variable number of short direct repeat sequences interspersed with nonrepetitive spacers. Strain-dependent "spoligotype" patterns of the *in-vitro*-amplified DNA are then determined after hybridisation with multiple spacer oligonucleotides, and represented as digital codes. The implementation of standardised protocols and nomenclature for identification and designation of spoligotypes and the establishment of online searchable databases for pattern matching has greatly enabled interlaboratory comparison of spoligotypes for strain tracking and epidemiology[2] (see Couvin *et al.*, 2019). Spoligotyping has often been combined with mycobacterial interspersed repetitive units (MIRU)-variable tandem repeat (VNTR) typing to increase the discriminatory power of MTBC genotyping. Currently a 24-loci MIRU-VNTR PCR assay is used for *M. tuberculosis* (Supply *et al.,* 2006) but optimisation of the combination of loci for a specific region is recommended for *M. bovis* and *M. caprae* to decrease the cost and time spent performing the assays whilst maximising discriminatory power.

1.3.3. Whole genome sequencing

The genomes of all members of the MTBC have been sequenced, and whole genome sequencing is increasingly being used routinely to genotype and distinguish between isolates for epidemiological studies as well as for understanding transmission chains and dynamics and MTBC evolution (Guimaraes & Zimpel, 2020).

2. Delayed hypersensitivity test

2.1. The intradermal tuberculin test

The intradermal tuberculin test is the standard method for the detection of tuberculosis in a wide variety of mammals, including bovine, ovine, caprine and cervid species. The test involves the intradermal administration of tuberculin purified protein derivative (PPD) and the measurement of increase in skin

2 www.mbovis.org; www.pasteur-guadeloupe.fr:8081/SITVIT2/index.jsp

thickness at the site of injection resulting from a delayed hypersensitivity reaction after a specified time interval (for instance, 72 hours in cattle).

The TST is not recommended for companion animals such as cats and dogs, where radiographic examination has supplanted its use.

The TST may be performed by single intradermal test (SIT) using PPD-B alone, either as the single cervical test (SCT) or the caudal (tail) fold test (CFT); or by the comparative cervical test (CCT) that measures the difference in increased skin thickness between PPD-B and that to PPD-A. The SIT typically exhibits greater sensitivity than the CCT, while the CCT provides greater specificity. The latter is often used to account for potential sensitisation of animals to environmental mycobacteria or antigenically cross-reactive organisms other than the members of MTBC (Good *et al*., 2018).

Recently developed defined antigen skin tests (DST) may overcome some of the limitations of SIT and CCT with improved sensitivity as compared with CCT and specificity as compared with SIT. DST may also be used to differentiate infected from BCG-vaccinated animals (Srinivasan *et al.,* 2019).

The SCT and CCT are performed in the mid-cervical region of bovine, ovine, caprine and cervid species, whereas the CFT is performed in the caudal fold of the tail (but is not suitable for cervids). The hypersensitivity responses to tuberculin observed in the cervical region are greater than in the caudal fold. However, both SCT and CFT have been successfully applied for the surveillance, control and elimination of tuberculosis in cattle in a number of countries.

Tuberculin skin tests are not well-validated in species other than bovids and cervids. As a consequence, the approaches to application and interpretation of the SCT, CCT and CFT in bovines are often considered as the default in other species. Alternate sites for antigen administration and interval for measurement of hypersensitivity responses may be indicated in some species. For example, in swine, it is administered at the base of the ear, and the hypersensitivity reaction evaluated after 48 or 72 hours. For non-human primates, "old tuberculin" is administered intrapalpebrally to the upper eyelid (see below, also Chapter 3.10.5 *Zoonoses transmissible from non-human primates*).

While animals exposed to MTBC organisms exhibit hypersensitivity to tuberculin, a positive tuberculin skin test result is unable to distinguish actively infected animals from those that may have recovered from infection. The test must therefore be interpreted in context.

Delayed hypersensitivity reactions may not develop for a period of 3–6 weeks following infection and recently infected or immunosuppressed individuals may present as false-negative. Hence, declaring a herd to be "free from tuberculosis infection" requires negative results from several sequential intradermal tests performed at 6–8-week intervals of all animals in the herd. In certain instances, anergy or hypo-responsiveness to tuberculin may occur in chronically infected animals with severe pathology leading to false negative results. In addition, animals may become desensitised due to repeated administration of tuberculin, in particular when applied at intervals of fewer than 6 weeks, and true positives may be missed. In such suspected false negative cases, serological or defined antigen cell-mediated immune response assays may be used as alternate or confirmatory assays.

The decision to use the SCT, CFT and CCT is dependent on the local regulations and the overall context and goals for performing the test. The CCT with higher specificity is typically applied for surveillance purposes or at the start of a control programme in an endemic country. Once the presence of the disease is confirmed a higher sensitivity approach may be preferred to avoid missing infected animals. CCT is also useful in regions with high environmental *Mycobacterium* exposure. In contrast, SCT or CFT with higher sensitivity are frequently applied to confirm freedom from infection in low burden settings.

2.2. Test procedure

2.2.1. Intradermal administration of PPDs

Personnel conducting the test should be specifically trained and certified for testing of animals. Prior to antigen administration, the injection sites must be clipped and cleaned. While performing the mid-cervical test, the thickness of the skin fold at the intended injection site should be measured with callipers, and the site suitably marked. All measurements of skin thickness should

be recorded in whole millimetres as measurements in fractions of millimetres may provide a false sense of precision. The same individual should measure the skin fold thickness before the injection and after the specified time interval using the same callipers to avoid additional introduction of operator or equipment-related variation.

A short needle, bevel edge outwards and graduated syringe charged with antigen is then inserted obliquely into the deeper layers of the skin to assure the delivery of specific volume of tuberculin. Preferably this should be performed using a calibrated and well-maintained multi-dose syringe or multiple injection gun. Because of the thin and sensitive skin of cervids, sheep, goats, and other species, a narrow gauge needle (e.g. 25G), may be preferred in some cases. The potency of both PPD-B and PPD-A antigens should be estimated by biological methods comparing with reference standard PPDs. A minimum 2,000 International Units (IU) of PPD should be administered in a volume not exceeding 0.2 ml. A correct injection is confirmed by visualising and palpating a small pea-like swelling at the site of injection.

In case of the CCT, the distance between the two injection sites should be approximately 15 cm: to prevent errors, routinely PPD-A is injected in the top site and PPD-B in the lower site. In young animals or smaller breeds with insufficient space to prepare the two injection sites on the same side of the neck, one injection may be performed at identical locations in the centre of the middle third of both sides of the neck.

In the CFT, 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 between the hairline and the ventral aspect of the fold. The injection site should be visually inspected closely and palpated carefully to detect changes from the normal.

The increase in skin-fold thickness at each injection site is then measured after 72 (± 4) hours interval or as prescribed depending on the species.

2.2.2. Interpretation of the test results in cattle

The interpretation of the skin test results is based on observation of clinical signs at the injection site and the recorded increase in skin thickness response following administration of antigen. Animals are considered as reactors when the increase in skin fold thickness exceeds a pre-specified threshold. The following guidelines may vary depending on the local context, and prevailing regulation. More stringent interpretations of test results, for example considering any palpable reaction as positive are permitted, and often recommended, e.g. in the CFT, to accelerate the local or national control programmes.

2.2.2.1. The single cervical test (SCT)

The single cervical test requires a single injection of PPD-B and the reaction is commonly considered to be negative if only limited swelling is observed, with an increase of 2 mm or less. The reaction is considered inconclusive if the increase in skin-fold thickness is more than 2 mm and less than 4 mm and considered positive if there is an increase of 4 mm or more.

ΔB	Interpretation of result
≤ 2mm	Negative
> 2 and < 4mm	Inconclusive
≥ 4mm	Positive

Animals that are inconclusive by the single intradermal test should be subjected to a second test after an interval of 6 weeks. Animals that repeatedly test as inconclusive should be considered as positive.

A more stringent interpretation is recommended, particularly in a high-risk population or in-contact animals.

2.2.2.2. The comparative cervical test (CCT)

In the interpretation of the CCT, a reaction to PPD-B is commonly considered negative if the increase in skin thickness is less than 2 mm. Depending on local legislation, the CCT is usually considered to be positive if the increase in skin thickness at the injection site of PPD-B is greater by more than 4 mm than the reaction shown at the site of the PPD-A. The reaction is considered inconclusive if the reaction to PPD-B is 2 mm or more, and is greater than the PPD-A reaction by 4 mm or less. All inconclusive reactors should be retested after 6 weeks. If the increase in skin thickness with PPD-B is less than or equal to the increase in that with PPD-A, the animal is classed as negative.

Animals that are inconclusive by the CCT should be subjected to a second test after an interval of 6 weeks. Animals that are positive or again inconclusive should be regarded as positive and be removed from the herd. To accelerate the clearance of TB from a known infected herd, a more stringent interpretation may be adopted wherein the inconclusive results may be interpreted as test positive in order to avoid the 6 weeks desensitisation waiting period. The presence/absence of clinical signs should also be considered in determining the herd status.

Interpretation of the comparative cervical test

First round of testing

ΔB (mm)	ΔB – ΔA (mm)	Interpretation of result
< 2	–	Negative
≥ 2	≤ 0	Negative
	> 0 and ≤ 4	Inconclusive
	> 4	Positive

Repeat testing inconclusives

ΔB (mm)	ΔB – ΔA (mm)	Interpretation of result
< 2	–	Negative
≥ 2	≤ 0	Negative
	> 0 and ≤ 4	Positive
	> 4	Positive

2.2.2.3. The caudal fold test (CFT)

The standard interpretation is that any swelling, sensitivity, or increase in skin thickness is considered to be a positive response to the bovine tuberculin. This interpretation may vary according to local regulations, for example in some countries an increase of >3mm may be used as the cut-off for positive reactions. The size of responses may vary and are not indicative of infectious status. Responses may be small, hard, pea-sized responses, diffuse responses, circumscribed responses, or large responses. If there is doubt about whether a response has occurred, the opposite side of the tail may be palpated to determine if there is a change from normal. Any observed change should be recorded. Test observation without palpation is unacceptable.

As is the case with the SIT, the use of the CFT as a screening test may result in false reactors to the test. Positive reactors to the CFT require secondary testing by using the CCT which must be performed within 10 days of the initial test, or else after 60 days.

2.2.3. Interpretation of test results in cervids

For cervid species (elk, reindeer, red deer, white tailed deer, sika, and others) only the mid-cervical test is used. In deer, both sites should be in the middle third of one side of the neck, the anterior site at least 100 mm behind the head and the posterior site approximately 130 mm from the other. In smaller deer, inoculation sites are used on each side of the neck.

Repeated skin tests should only be carried out at a 120-day interval to minimise desensitisation. In the EU, the same interpretation as for bovines is used in cervids for both the SCT and CCT. In the United States, the SCT is recommended as a primary test, and the CCT as a supplemental test in captive cervids. If an animal reacts to the SCT, a retest with the CCT after 120 days is indicated.

2.2.4. Interpretation of test results in sheep and goats

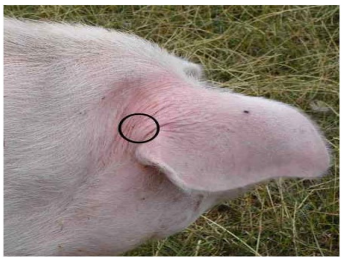
The skin test and its interpretation are less well validated in sheep and goats than in cattle and deer (see Roy *et al.*, 2020). The skin test can be performed in both species in the mid-cervical or the scapular area using the SCT or CCT or in the caudal fold. Other areas free of wool can be used, e.g. the upper inner areas of the rear legs, which avoids loss of wool quality. Given the small size of the neck in both species, the CCT is often performed by applying the PPDs on opposite sides of the neck. Evaluation of the SCT and CCT can be done by measuring and applying the same interpretation as for cattle, depending on the national legislation.

2.2.5. Interpretation of test results in camelids

The skin test can be performed at the axillary, cervical or prescapular regions. When both PPD-B and PPD-A are used, they should be injected intradermally on each side at identical sites (cervical, prescapular or axillary). In the cervical and prescapular sites, it may be possible to inject both PPDs on the same side if separated sufficiently. Evaluation of the SCT and CCT can be done by measuring and applying the same interpretation as for cattle, depending on national legislation.

Skin tests for camelids have shown lower sensitivity and specificity compared with that for cattle. In the EU, it is recommended to use as a complementary test to the serological protocol.

2.2.6. Interpretation in pigs



It is advisable to use the CCT with a dose of 2000 IU of each antigen. Reactions in pigs may be very pronounced and higher concentrations of PPDs may result in necrosis of the skin. The preferred site is the loose skin at the dorsal surface of the ear at the furrow between head and ear. The PPD-A and PPD-B are injected at the base of the left and right ear, respectively. The reaction can be read after 48 or 72 hours. Interpretation should follow either the same scheme as used for the CCT in cattle or by palpation, depending on local legislation.

2.2.7. Interpretation in exotic and zoo animals

For non-human primates, "mammalian old tuberculin" is administered intrapalpebrally in the middle of the upper eyelid adjacent to the edge of the lid, to detect tuberculosis caused by *M. tuberculosis* or *M. bovis.* If repeat testing is required, the eyelid should be alternated between each test. The readings are taken at 24, 48 and 72 hours post-injection. The reactions of grade 1 and 2 are negative, grade 3 inconclusive and grades 4 and 5 are interpreted as positive. See also chapter 3.10.10 *Diseases transmissible from non-human primates.*

Grade	Reaction
0	No Reaction
1	Bruise – extravasation of blood in the eyelid associated with the injection of tuberculin
2	Varying degrees of erythema of the palpebrum
3	Moderate swelling with or without erythema
4	Obvious swelling of the palpebrum with drooping with or without erythema
5	Necrosis of the eyelid with varying degrees of swelling, including eyelid partially or completely closed

Intradermal tuberculin tests are not recommended for use in elephants due to a high false-negative rate in culture-positive animals.

2.3. Defined antigen skin tests

Research directed toward identification and characterisation of the key antigenic components of field isolates of *M. bovis* and BCG vaccine strains has led to development of skin tests and blood tests that are based on detection of responses to molecularly defined antigens (Middleton *et al.*, 2021). These defined antigen diagnostic tests could potentially be utilised on their own or in combination with other tests, including conventional PPD tuberculin tests, to improve diagnostic sensitivity or specificity, as well as to

help differentiate *M. bovis*-infected animals from those which have been vaccinated or exposed to environmental mycobacteria (Srinivasan *et al.*, 2019).

3. Blood-based laboratory tests

Besides the intradermal tuberculin test, a number of blood tests that measure cellular or humoral immune responses of animals to MTBC have been developed. Due to cost and or complexity considerations these laboratory-based assays are often used as ancillary tests to improve detection of infected animals (parallel testing), or to confirm results of an intra-dermal skin test (serial testing). Administration of tuberculin skin tests is known to enhance the sensitivity of antibody-based tests, if serum is taken between 2-8 weeks after skin testing, leading to greater test accuracy. The IGRA measures cellular immunity while humoral antibodies are measured by serological methods such as ELISA and lateral flow assays.

3.1. Interferon-gamma release assay (IGRA)

In this test, the release of a lymphokine gamma interferon is measured in a whole-blood culture system. The assay is based on the release of interferon-gamma from sensitised lymphocytes during a 16- to 24-hour incubation period with specific antigen (PPD-tuberculin) (Wood *et al*., 1990). The test makes use of the comparison of interferon-gamma production following stimulation with avian and bovine PPD. The detection of interferon-gamma is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine interferon-gamma. It is recommended that the blood samples be transported to the laboratory avoiding extreme temperatures (e.g. a range of 17–27°C) and the assay set up as soon as practical, but not later than the day after blood collection (Coad *et al*., 2007). In some areas, especially where 'non-specificity' is prevalent, some concerns about the accuracy have been expressed when blood stimulation is performed with PPDs. However, because of the capability of the IGRA to detect early infections, the use of both IGRA and skin 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 (Gormley *et al*., 2006). The use of defined mycobacterial antigens such as ESAT-6 and CFP-10 can improve the specificity (Buddle *et al*., 2001), and these antigens are employed in a number of countries such as the United Kingdom, New Zealand and France for serial testing. The use of such antigens may also offer the ability to differentiate BCG-vaccinated from unvaccinated animals. In animals that are excitable, difficult or dangerous to handle, the advantage of the IGRA over the skin test is that the animals need be captured only once. The IGRA has been approved for use in a number of national programmes including the EU, UK, USA, New Zealand, and Australia. In New Zealand and the United Kingdom for example, the IGRA 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.

3.2. Serology for detection of specific antibodies

The application of serological assays for diagnosis of tuberculosis in animals has increased in the 21st century. ELISA is the most widespread technique, however alternative serological platforms have been developed (e.g. lateral flow tests, a multi-antigen print immunoassay or a multiplex chemiluminescent assay) (Bezos *et al*., 2014). Serological assays have been proposed as a valuable ancillary diagnostic tool to complement cell-based methods, increasing the detection of infected animals and helping to control tuberculosis in domestic and wild animals (Casal *et al*., 2017; Thomas & Chambers, 2021). In camelids it is recommended to use serology as a complementary test to the skin test and 15 to 30 days after skin testing. The advantages of these tests are their simplicity, low cost and lower logistical demands compared, for example, with the IGRA, as they do not require immunological stimulation with antigens, and samples can be stored for a prolonged time before processing. These tests could be especially helpful for detecting anergic animals that do not respond well to cell-based immune techniques. Their sensitivity, however, is lower than cell-based tests, particularly in recently infected animals, increasing in advanced stages of the disease. The use of combinations of specific antigens such as MPB83, MPB70, ESAT-6 and CFP-10 has been demonstrated to increase the sensitivity and specificity of serological tests (Thomas & Chambers, 2021).

In recent years, new antibody detection tests using different methodologies have also demonstrated better performance than skin tests in wildlife (wild boar, deer, badgers) and domestic animals (cattle, sheep, goats, alpacas, pigs). Examples include the P22 ELISA based on a multiprotein complex named P22 obtained by affinity chromatography from PPD-B, or a double-recognition ELISA that detects

specific antibodies against MPB83, using MPB83 protein as both an antigen coating the plate and as a conjugate (Cardoso-Toset *et al*., 2017; Casal *et al*., 2017; Infantes-Lorenzo *et al*., 2019). Moreover, the booster effect observed on the antibody titres caused by a recent intradermal tuberculin test in MTBC-infected animals can be used as an valuable diagnostic strategy to increase the sensitivity of serological tests at 15–30 days after PPD injection (Casal *et al*., 2014).

Serological tests are valuable for detecting MTBC infections in wildlife. A lateral flow test based on MPB83 protein and CFP10/ESAT-6 fusion protein, has been shown to be useful for detecting infection in domestic animals, but particularly in wildlife and zoo animals because it is easy to perform and gives immediate test results. Although their sensitivity is limited, these methods are useful where no cell-based tests are available and where skin testing has proven unreliable, (Greenwald *et al*., 2009; Lyashchenko *et al*., 2008; Thomas & Chambers, 2021).

WOAH has evaluated a number of tests for use in cattle serum and plasma as supplemental tools, together with other methods, for diagnosing and managing tuberculosis infection. See the WOAH Register of Diagnostic Kits for further information[3].

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Vaccines

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

The only currently available vaccine against *M. bovis* infections is bacille-Calmette-Guerin (BCG), a live attenuated strain of *M. bovis*. Trials have been conducted on a number of other vaccines, but none has been shown to induce a superior protection to BCG (Vordermeier *et al.,* 2016).

BCG has been shown to be safe in cattle and some other livestock as well as in free-living and captive wildlife. Studies have noted variable levels of efficacy in experimental and field trials in cattle. This variability has been attributed to factors including vaccine formulation, age of animal, route of vaccination, and exposure to environmental mycobacteria. The protection provided by BCG is due to a reduction in susceptibility of vaccinates to infection, as well as through reduction of onward transmission from vaccinated animals resulting from lowered pathology and infectiousness.

Similarly, to what is observed in humans, a systematic review and meta-analysis showed only modest (~25%) direct efficacy of BCG against bovine TB challenge in cattle (Srinivasan *et al.,* 2021). However, scenario analyses considering both direct and indirect effects suggest that disease prevalence could be substantially reduced up to official TB-free depending on the starting levels of infection, and 50–95% of cumulative cases averted over 50 years with BCG vaccination. For these reasons, BCG vaccination may help accelerate control of bTB in endemic settings, particularly with early implementation in the face of dairy intensification in regions that currently lack effective bTB control programs.

Experimental trials have established that protection wanes between one to two years post-vaccination, but revaccination when immunity has waned after two years boosted protection. BCG vaccination of infected cattle does not result in exacerbation of infection (Buddle *et al.,* 2016).

BCG vaccine strains including Danish 1331, Pasteur 1173P2 and Russia have been used at a dosage ranging from 10^4 to 10^6 colony-forming units (CFU) given subcutaneously. Since the use of vaccines may compromise tuberculin skin tests or other immunological tests, the use of molecularly defined tuberculin as diagnostic antigen is needed. 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 (Vordermeier *et al.,* 2016) or as skin test reagents (Srinivasan *et al.,* 2019) and are based on the use of gene products that are encoded on *M. bovis* gene regions that are deleted or not expressed in some BCG strains. Hence, feasibility of

3 https://www.woah.org/en/what-we-offer/veterinary-products/diagnostic-kits/the-register-of-diagnostic-kits/

implementation of BCG vaccination in cattle and other livestock species may be enabled by availability of DIVA tests once fully validated and the regulatory frameworks established.

BCG vaccines may also be used to reduce the spread of *M. bovis* in wildlife reservoirs of infection. BCG has been granted a limited regulatory approval in the UK for intramuscular vaccination of badgers at a dose of $2–8 \times 10^6$ CFU. Field trials have found that BCG significantly reduces infection from natural exposure to *M. bovis* in badgers, reduces the risk of infection in unvaccinated cubs in vaccinated social groups (Carter *et al.,* 2012) and in some cases may result in a lowering of cattle TB incidence (Martin *et al.,* 2020). Oral dosing of BCG to brush-tailed possums and European badgers in field trials have been shown to confer significant protection against natural exposure to *M. bovis* (Gormley *et al.,* 2017; Tompkins *et al.,* 2009). Oral dosing of possums and badgers with BCG vaccine has been shown to be safe, although transient shedding of BCG in faeces has been demonstrated (Perrett *et al.,* 2018).

C2. Production of bovine tuberculin

Tuberculins are prepared from the heat inactivated liquid cultures of MTBC grown as pellicles on broth. These PPDs, precipitated using trichloroacetic acid (TCA) or ammonium sulphate, replaced the former heat-concentrated tuberculins, having a higher specific potency. Additional filtration steps to remove non-specific cell wall material further increased the specificity. In addition, a mutant of *M. bovis,* named AN5, that showed "luxurious" growth as a pellicle on glycerol broth was selected to facilitate large scale economic production of PPD-B.

PPD-B produced from cultures of *M. bovis* AN5 was subsequently shown to be more specific in the intradermal assay in cattle than "human" PPD prepared from cultures of *M. tuberculosis* that had been used previously in eradication campaigns.

In the interests of standardisation, the *Terrestrial Manual* will continue to recommend use of PPD-B in the SCT, or in combination with PPD-A in the CCT, as the preferred reagent for intradermal skin testing and/or the IGRA in regions where BCG vaccination is not being considered. See Sections B.2 *Delayed hypersensitivity test* and B.3.1 *Gamma-interferon assay* for details of the test procedures. All PPDs used for national or local surveillance programmes should be validated and calibrated in comparison with established reference standards.

2.1. Seed management

As the quality of PPD-B largely depends on the ability of the production strain to produce dominant T-cell antigens like ESAT-6, CFP-10 and Rv3615c as well as the ability to grow on glycerol broth, other virulent and well characterised *M. bovis* strains could be suitable for the production of PPD-B. However, in the interests of international standardisation and harmonisation of production and potency testing it is recommended that defined isolates of *M. bovis* AN5 or *M. bovis* Vallee should be used for PPD-B production. These have been in use for over 70 years and are well characterised. A record must be kept of their origins and subsequent history, including the number of passages from the original receipt from a Reference Laboratory (e.g. for AN5 since supplied by "Weybridge"), a spoligotype pattern and (where possible) whole genome sequence data. The strains of *M. bovis* used as seed cultures must be shown to be free from contaminating organisms.

2.2. Method of culture

If the source culture for production was a primary isolate grown on solid medium, it is necessary to adapt the organism to grow as a floating culture or pellicle (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 pellicle growth on the liquid medium, it may be used to produce the Master Seed lot, which is preserved in freeze-dried form. All seed lots should be checked in a pilot study for their ability to support the production of a PPD-B of sufficient quality.

In accordance with GMP guidelines and to guarantee a constant quality of PPD-B, a Seed Lot System of Master and Working seeds for the well-characterised production strain will have to be established and stored at -70°C or below. The Working Seeds used to inoculate the production media must not be more than four culture passages from the Master Seed. As the Working Seeds lose the ability to produce a suitable PPD-B, they should be replaced at frequent intervals with new Working Seeds.

2.3. Method of manufacture and in-process control

For the production of PPD-B, Working Seeds are inoculated on glycerol broth (made from beef guaranteed free of TSEs) in Roux culture flasks and when a pellicle has been formed on top of the liquid medium, this pellicle will be divided and used to inoculate a larger number of penicillin flasks containing a synthetic production medium, e.g. Dorset-Henley, to avoid non-*M.bovis* proteins contaminating the final product.

During growth, which will take approximately 60 days, growth should be checked daily and when "abnormal" cultures are detected, e.g. showing changes in colour, signs of contamination, or sinking pellicles, these should be removed since they will negatively affect the quality of the final product. These abnormal cultures should be discarded after autoclaving.

Subsequently, the cultures are heat inactivated at 98–100°C for 2–3 hours and cellular debris is removed in a series of filtration steps, starting with coarse filters to remove larger particles and finally clarified using for example a Seitz series of filters with EK1 as the final sterilisation filter. The combination of different filters and their respective cut-off's as well as the number of each filter used depends largely on the volume to be processed but the removal of as much insoluble material is an essential step to maximise the specificity of the final product.

Proteins in the filtrate are precipitated by TCA (or ammonium sulphate depending on the producer). Precipitation is followed by a series of washing and centrifugation steps then alkaline treatment to bring the water soluble proteins to a neutral pH (6.5–7.0) in an isotonic glucose-phosphate buffer containing 0.04% w/v phenol.

The protein concentration of the final product or "concentrate" will be determined by the Kjeldahl method and is routinely presented as total nitrogen and TCA precipitable nitrogen.

The potency and specificity of the final product is determined in the guinea pig assay using the International standards for PPD-B and PPD-A, respectively.

Depending on the outcome of the potency assay, final dilutions are made for the commercial product with the same isotonic glucose-phosphate buffer pH 6.5–7.0 containing 0.04% w/v phenol, to obtain a minimal dose of 2000 IU in the intradermal assay. Currently, many commercially available PPD-Bs contain an estimated potency of 2500 or 3000 IU per dose to optimise sensitivity of the intradermal assay.

For the specificity of the PPD-B it is essential that the final protein concentration is kept as close as possible to 1.0 mg/ml, products with a higher protein concentration are known to be less specific, hence many countries demand a maximum protein concentration as part of their regulatory requirements.

2.4. 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.

2.4.1. Sterility

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

2.4.2. 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.

2.4.3. 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 PPD-B sample 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 PPD-B sample. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

2.4.4. Potency

In the 1980s the World Health Organization (WHO) developed and designated a new international standard to which it assigned a unitage of 32,500 IU/mg, which gave it equivalency to earlier local standards. This became known as the Bovine International Standard (BIS) tuberculin. This is available from the National Institute for Biological Standards and Control in the UK, for use by Veterinary Services and commercial manufacturers to calibrate national and corporate reference standards to ensure that production batches conform to international standards. As supplies of BIS are now limited, WOAH has initiated an international collaborative study to produce and calibrate a new reference reagent, to be known as the International Standard Bovine Tuberculin-2 (ISBT-2). An announcement will be made by WOAH when its validation has been completed.

Countries where bovine PPD tuberculin is produced should establish their own national reference preparations for bovine PPD as working standards. These national reference preparations should be calibrated in guinea-pigs against the official international standard (BIS/ISBT-2) for bovine PPD.

2.4.4.1. Standardisation of bovine PPD

a) Guinea-pigs sensitised with live *M. bovis*

The potency of a PPD-B sample is determined by comparison with a reference preparation of PPD-B, either the international standard or a derived national reference standard, in guinea-pigs infected with the production strain used e.g. *M. bovis* AN5.

The model, including variables such as breed and supplier of animals, infection dose, duration of infection needs to be validated prior to performing the assay: the combination of these variables and the expected potency of the serial dilutions of the PPD-B samples should result in lesions with a diameter of not less than 8 mm and not more than 25 mm for the assay to be valid. Infection doses can differ considerably between batches with regard to their virulence and each new batch will have to be carefully tested to avoid animal welfare issues as well as open tuberculosis which would result in excretion of bacilli and thus an increased health risk for the operators.

At least 8 albino guinea-pigs, each weighing 400–600 g, are infected with a low dose, e.g. 0.0001 mg wet mass of live bacilli of the homologous production strain e.g. *M. bovis* AN5 not less than 4 weeks prior to the assay. The bacilli are suspended in 0.5 ml of a 9 g/litre solution of sodium chloride and a deep intramuscular injection is made on the medial side of the thigh.

A suitable design for a potency assay is as follows: The potency of two PPD-B samples is estimated in the above infection model of 8 or 9 guinea-pigs against the standard for PPD-B, with three dilutions at five-fold intervals of each PPD-B, the 2 samples as well as the standard. The dilutions of the tuberculin preparations are made in isotonic phosphate-buffered saline (pH 6.5–7.5) containing 0.005 g/litre of polysorbate 80. The optimal concentrations are chosen in such a way that good readable skin reactions

are obtained with acceptable (8–25 mm) limits. As an example: amounts of 0.001, 0.0002 and 0.00004 mg PPD-protein corresponding to the international standard for PPD of 32, 6.4 and 1.28 IU, respectively, can be used. The injection volume is 0.2 ml.

In a single assay, two test PPDs are compared with the standard PPD in nine guinea-pigs, applying eight intradermal injections per animal and employing a balanced incomplete Latin square design.

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 calipers in tenths of a millimetre and recorded on assay sheets. The results are statistically evaluated using standard statistical methods for parallel-line assays according to Finney (1978). A statistical program for the parallel line analysis, Combistats, is available from the European Pharmacopeia or EDQM.

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 test is not valid unless the confidence limits (p = 0.95) are not less than 50 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 66 per cent and not more than 150 per cent of the stated potency. The stated potency is not less than 20,000 IU/ml.

b) Guinea-pigs sensitised with killed *M. bovis*

For laboratories that do not have biosecure facilities to house guinea-pigs infected with *M. bovis,* an alternative potency assay is often used, using heat-inactivated antigen to sensitise guinea-pigs. Because of differences in the sensitising antigens, results are not directly convertible between the two models. It is advised that interlaboratory comparisons are conducted to evaluate the potency of tuberculins assayed by the two methods.

As with the live AN5 assay, the "heat-inactivated" tuberculin potency assay should be validated to optimise the combination of sensitising dose and the potency of the PPD-B dilutions assayed in the model.

The assay is performed as follows: the PPD tuberculin is bioassayed in guinea-pigs sensitised with heat inactivated *M. bovis*, of the same strain used for PPD production, 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 PPD-Bs are compared with international/national standard PPD-B 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 heat inactivated bacilli are suspended in buffer and made into an emulsion with mineral oil adjuvant. A deep intramuscular injection is made on the medial side of both thighs, using a dose of 0.5 ml.

2.4.5. 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 975 and 39 IU, are chosen because these doses give good readable skin reactions. The injection doses of the standard are lower, namely 0.001 mg and 0.00004 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 the same as with the potency test, but the interpretation should take into account that a satisfactory PPD should have a biological activity that does not surpass 10% of a standard with homologous sensitisation.

2.4.6. 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.

2.4.7. pH control

The pH should be between pH 6.5 and 7.5.

2.4.8. Protein content

The protein content is determined as indicated in Section C.2.*3 In-process control*.

2.4.9. 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.

2.4.10. 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).

2.4.11. Precautions (hazards)

Appropriately diluted tuberculin injected intradermally in humans or animals, can result in a localised reaction at the injection site. Even in very sensitive individuals, severe, generalised reactions are extremely rare and limited. Operators with known sensitivity to tuberculin should carry out a risk assessment before handling the material.

C3. Production of avian tuberculin

Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated culture filtrate of *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity to identify birds infected with or sensitised to the same species of *Mycobacterium*. Importantly it is also used to aid differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis. An international standard preparation of PPD-A is being developed by WOAH to replace the former WHO Standard[4].

The general principles in Chapter 1.1.8 *Principles of veterinary vaccine production,* should be followed for injectable diagnostic biologicals such as tuberculin. The standards here and in chapter 1.1.8 are intended to be general and may be supplemented by national and regional requirements.

3.1. Characteristics of the seed culture

3.1.1. Biological characteristics of the master seed culture

Strains of *M. a. avium* used to prepare seed cultures should be purchased from a culture collection and identified as species by appropriate tests. Several strains are recommended for

4 PPD of *M. avium* tuberculin, WHO (1955) Technical Report Series, no.96, 11.

this purpose in different countries. For example, in the European Union, D4ER and TB56 are recommended. The relevant national recommendations should be followed. Globally there are commercial sources of PPD-A.

3.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Seed cultures should be shown to be free from contaminating organisms and to be capable of producing tuberculin of sufficient potency. The necessary tests are described below.

3.2. Method of manufacture

3.2.1. Procedure

The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid's medium). When the culture has been adapted to a liquid medium, it can be maintained by a passage at 2–4-week intervals (Angus, 1978; Haagsma & Angus, 1995).

The organism is cultivated in modified Dorset-Henley's synthetic medium, then killed by heating in flowing steam and filtered to remove cells. The protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid are used), washed, and resuspended. 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. Mercurial derivatives should not be used. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile neutral glass containers, then sealed to prevent contamination. The product may be freeze-dried.

3.2.2. Requirements for ingredients

The production culture substrate must be shown to produce a product that conforms to the European Pharmacopoeia (2024[5]) standards or other international standards such the WHO (WHO, 1987). It must be free from ingredients known to cause toxic or allergic reactions.

3.2.3. In-process controls

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-A tuberculin, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be pH 6.6–6.7. The Kjeldahl method determines the protein level (total organic nitrogen) of the PPD-A concentrate. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

3.2.4. Final product batch tests

i) Sterility

Sterility testing is generally performed according to the European Pharmacopoeia (2024) or other guidelines (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

ii) Identity

One or more batches of tuberculin may be tested for specificity together with a standard preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis* using a procedure similar to that described in Section C3.3.2.4.iv. The potency of the preparation of avian tuberculin must be shown to be not more than 10% of the potency of the standard preparation of bovine tuberculin used in the potency test. The

5 https://www.edqm.eu/en/d/234640?p_l_back_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative

use of animals for this purpose should be reviewed and approved by your institution's ethical committee.

iii) Safety

Tuberculin PPD-A can be examined for freedom from living mycobacteria using the culture method described previously. This culture method, which does not require the use of animals, is used in many laboratories, and its use is encouraged over the use of animals for this purpose.

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 injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, 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 examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture. Each filled container must be inspected before it is labelled, and any showing abnormalities must be discarded.

A test for the absence of toxic or irritant properties must be conducted according to the European Pharmacopoeia (2024) specifications or the equivalent regulatory documents for each country or region.

To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test are each injected intradermally on three occasions with the equivalent of 500 International units – one IU is equal to the biological activity 0.02 μg of PPD – of the preparation under test in a 0.1 ml volume. In the USA and Canada, the potency of the tuberculin is expressed as tuberculin unit (TU) rather than IU. One TU is also defined as 0.02 μg of PPD. Each guinea-pig, together with the 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 tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

iv) Batch potency

The potency of avian tuberculin is determined in guinea-pigs sensitised with *M. a. avium* compared with a standard preparation calibrated in IU or TU.

Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering a suitable dose of inactivated or live *M. a. avium* to each by deep intramuscular injection. The test is performed between 4 and 6 weeks later. Briefly, have the guinea-pigs' flanks shaved (an area large enough to provide space for three-to-four injections on each side). Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in an isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the dilutions to the injection sites randomly using a Latin square design. The dilutions correspond to 0.001, 0.0002, and 0.00004 mg of protein in a final dose of 0.2 ml, injected intradermally.

At 24 hours, the reactions' diameters are measured, and the results are calculated using standard statistical methods, taking the diameters to be directly proportional to the logarithms of the concentrations of the tuberculin. The estimated potency must be not less than 75% and not more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of error ($p = 0.95$) are not less than 50% and not more than 200% of the estimated potency. If the batch fails a potency test, the test may be repeated one or more times, provided that the final estimate of potency and fiducial limits is based on the combined results of all the tests.

It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml or approximately 0.5 mg protein per ml, giving a dose for practical use of 2500 IU/0.1 ml.

3.3. Requirements for authorisation/registration/licensing

3.3.1. Manufacturing process

The manufacturing process should follow the requirements of European Pharmacopoeia (2024) or other international standards.

3.3.2. Safety requirements

3.3.2.1. Target and non-target animal safety

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). The pH should be between 6.5 and 7.5.

3.3.2.2. Precautions (hazards)

Experience 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 persons, severe, generalised reactions are extremely rare and limited.

3.3.3. Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (not exceeding 25°C) and protected from light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

Following accepted practice, tuberculin should be stored at a temperature of between 2°C and 8°C and protected from light; they may be used up to the end of the expiry date as specified in the licence for production of tuberculin. Recent research on the temperature stability of human, bovine, and avian tuberculin solutions has shown that they are stable for a year at 37°C. This should be further explored as these products are used in the field in remote areas of the world where maintaining temperature control is very difficult (Maes *et al.,* 2011).

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NB: There are WOAH Reference Laboratories for mammalian tuberculosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for mammalian tuberculosis

NB: FIRST ADOPTED IN 1989 AS BOVINE TUBERCULOSIS. CHAPTER FIRST ADOPTED WITH CURRENT TITLE IN 2022.