**USA COMMENTS IN RED FONT**

Chapter 3.3.6.

avian TuberculoSIS

SUMMARY

**Description of the disease:** Avian tuberculosis, or avian mycobacteriosis, is ~~an important~~ a significant disease that affects companion, captive exotic, wild, and domestic birds and mammals. The disease is most often caused by Mycobacterium avium subsp. avium (M. a. avium), a member of the M. avium complex. However, more than ten other mycobacterial species have been reported to infect birds. The most significant cause of poultry disease is M. a. avium.

Clinical signs of the disease vary depending on the organs involved. The classical presentation is characterised by chronic and progressive wasting and weakness. Diarrhoea ~~is common~~ and joint swelling are standard features in infected flocks. Some birds may show respiratory signs, and occasionally, sudden death occurs. Some birds may develop granulomatous ocular lesions.

Mycobacterium tuberculosis, the agent that most commonly causes human tuberculosis (gene IS6110) is ~~less commonly~~ rarely the cause of infection in birds, and it is often ~~as a~~ the result of transmission from pet bird owners or caretakers of captive birds.

Members of M. avium complex: M. a. avium (serotypes 1–3; containing ~~gene segments~~ insertion sequences IS901 and IS1245), M. avium subsp. hominissuis (serotypes 4–6, 8–11, and 21; lacking gene segment IS901 and containing segment IS1245) and M. intracellulare (serotypes 7, 12–20, and 22–28; lacking both IS901 and IS1245) can also infect an extensive range of mammals such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species. In humans, all members of the M. avium complex and M. genavense ~~are capable of inducing~~ can induce a progressive disease that is refractory to treatment, ~~mostly~~ mainly in immunocompromised patients.

~~All manipulations involving~~ Due to the contagious nature of this group of organisms, handling ~~of~~ open live cultures or ~~of~~ material from infected birds must only be carried out ~~with~~ after an appropriate ~~biorisk management~~ risk assessment and the implementation of biosafety measures designed to avoid infection.

Diagnosis of avian tuberculosis in birds depends on the demonstration of ~~the above-mentioned~~ a mycobacterial species in live or dead birds or the detection of an immune response, cellular or humoral, culture examination, or gene segments ~~IS6110, IS901 and IS1245~~ by polymerase chain reaction (PCR) in the excretions or secretions of live birds.

**Detection of the agent:** Where clinical signs of avian tuberculosis are seen in the flock, or typical tuberculous lesions are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a quick positive diagnosis. If acid-fast bacilli are not found but typical tuberculous signs or lesions are present in the birds, a culture of the organism or PCR must be attempted~~. PCR could also be carried out directly~~ on tissue samples. Any acid-fast organism isolated should be identified by nucleic-acid-based tests or chromatographical (e.g. high-performance liquid chromatography [HPLC]) criteria. Serotyping of isolates of M. avium complex members or PCR for 16S rRNA gene followed by sequencing, or the presence of an amplicon for the insertion sequences IS6110, IS901, and IS1245 ~~could~~ can also be performed. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a valuable tool as well for isolates following culture

**QUESTION FOR THE BIOLOGICAL STANDARDS COMMISSION:** IS6110 is associated with Mycobacterium tuberculosis complex (MTBC), not Mycobacterium avium complex (MAC). Is its inclusion to rule out or differentiate for MAC as birds can rarely be infected with MTBC?

**Tuberculin ~~test~~ and serological tests:** These tests are ~~normally~~ typically used to determine ~~the~~ disease prevalence ~~of disease~~ in a flock or ~~to~~ detect infected birds. When used to detect the presence of avian tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.

In domestic fowl, the tuberculin test in the wattle is the test of choice. This test is less useful in other species of bird. ~~A better test, especially in waterfowl, is~~ The whole blood stained-antigen agglutination test is better, especially in waterfowl. It is more reliable and ~~has the advantage that it will~~ can give a result within a few minutes while the bird is still being held.

**Requirements for vaccines and diagnostic biologicals:** No vaccines are available for use in birds. Avian tuberculin purified protein derivative ~~(PPD)~~ is the standard preparation for use in the tuberculin test of domestic poultry. Avian PPD is also used as a component in the comparative intradermal tuberculin test in cattle (see Chapter 3.1.13 Mammalian tuberculosis [infection with Mycobacterium tuberculosis complex]).

A. introduction

Several mycobacterial species can be involved in the aetiology of avian tuberculosis ~~and~~, also known as avian mycobacteriosis. Avian tuberculosis is most commonly ~~produced~~ caused by infection with *Mycobacterium avium* subsp. *avium* (serotypes 1, 2, and 3: containing specific gene segment IS*901* and nonspecific segmentIS*1245*) and less frequently by *M. genavense* (Guerrero *et al.*, 1995; Pavlik *et al.*, 2000; Salamatian *et al.,* 2020; Sattar *et al.,* 2021; Tell *et al*., 2001). Avian mycobacteriosis is also caused by other two members of the *M. avium* complex: *M. avium* subsp. *hominissuis* (serotypes 4–6, 8–11, and 21: lacking gene segment IS*901* and containing segment IS*1245* and mainly infecting humans and pigs)and *M. intracellulare* (serotypes 7, 12–20, and 22–28: lacking both gene segments IS*901* and IS*1245*) and by *~~M. intracellulare~~*~~,~~ *~~M. scrofulaceum~~*~~,~~ *~~M. fortuitum,~~* ~~and~~ other potentially pathogenic mycobacterial species including *M. scrofulaceum* and *M. fortuitum*. Under some circumstances, an extensive range of mammalian species, such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic animals, can be infected by these mycobacterium species (Dvorska *et al.*, 2004; Kunze *et al.,* 1992; Mijs *et al.*, 2002; Shitaye *et al.*, 2009; Tell *et al*., 2001; Thorel *et al*., 1997; 2001). *Mycobacterium tuberculosis* and *M. bovis* are ~~less common as causal~~ rarely the causative agents of tuberculosis in birds (Hoop, 2002; Lanteri *et al.,* 2011; Peters *et al.,* 2007; Schmidt *et al.,* 2022; Tell *et al*., 2001).

*Mycobacterium avium* species with standing in nomenclature as of 2023[[1]](#footnote-2) (Arahal *et al.,* 2023) consists of ~~four~~ three subspecies: *M. avium* subsp. *avium*, *~~M. avium~~* ~~subsp.~~ *~~hominissuis~~*~~,~~ *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis* (Mijs *et al.*, 2002; Thorel *et al*., 1990). The latter is the causal agent of Johne’s disease, or paratuberculosis, in ruminants and other mammalian species (see Chapter 3.1.16 *Paratuberculosis* [*Johne’s disease*]). *Mycobacterium a. silvaticum,* which like *M. avium* subsp. *paratuberculosis* grows *in-vitro* only on media with Mycobactin, which can cause avian tuberculosis in wood pigeons (Thorel *et al.*, 1990). With the widespread use of whole genome sequencing (WGS) and bioinformatics, some studies have investigated the classification of species belonging to the genus *Mycobacterium* and have proposed that *M. avium* comprises three subspecies *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *lepraemurium*. Further subdividing *M. avium* subsp. *avium* into three variants *M. avium* subsp. *avium* var. *avium*, *M. avium* subsp. *avium* var. *silvaticum*, and *M. avium* subsp. *avium* var. *hominissuis* (Riojas *et al.,* 2021; Tortoli *et al.,* 2019).

~~All~~ *~~M. a avium~~* ~~isolates from birds and mammals, including humans, have a multiple repetitive sequence IS~~*~~901~~* ~~in their genome and produce a characteristic three-band pattern in IS~~*~~1245~~* ~~restriction fragment length polymorphism (RFLP) as described and standardised previously (Dvorska~~ *~~et al.~~*~~, 2003; Ritacco~~ *~~et al~~*~~., 1998). This repetitive sequence is also present in~~ *~~M. a. silvaticum~~* ~~and RFLP analysis can help with identification. IS~~*~~901~~* ~~has only been detected in~~ *~~M. avium~~* ~~strains with serotypes 1, 2 and 3 (Pavlik~~ *~~et al~~*~~., 2000; Ritacco~~ *~~et al~~*~~., 1998) that are apparently more pathogenic to birds than other serotypes (Tell~~ *~~et al~~*~~., 2001). On the basis of genetic and phenotypic differences it has recently been proposed to differentiate~~ *~~M. a. avium~~* ~~into two subspecies based on the target organism:~~ *~~M. a. hominissuis~~* ~~for human and porcine isolates and~~ *~~M. a. avium~~* ~~for bird-type isolates (Mijs~~ *~~et al~~*~~., 2002).~~ *~~Mycobacterium a. hominissuis~~* ~~has polymorphic multiband IS~~*~~1245~~* ~~RFLP patterns and is able to grow between 24 and 45°C (Mijs~~ *~~et al~~*~~., 2002; Van Soolingen~~ *~~et al.~~*~~, 1998). It is worth noting that the typical features of bird-isolates, the three-band pattern in IS~~*~~1245~~* ~~RFLP and presence of IS~~*~~901~~*~~, have also been found in cervine and bovine isolates of~~ *~~M. a. avium~~*~~.~~

Avian tuberculosis in birds is most prevalent in gallinaceous poultry and ~~in~~ wild birds raised in captivity. Turkeys are ~~quite~~ susceptible, but ducks, geese, and other water birds are comparatively resistant. The practices of allowing poultry to roam at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of the causal agent of avian tuberculosis among them. Infected individuals and contaminated environments (water and soil) are the ~~main~~ primary sources of infection. The above-mentioned mycobacterial species causing avian tuberculosis can survive for several months in the environment (Dvorska *et al.*, 2007; Kazda *et al.*, 2009; Shitaye *et al.*, 2008; Tell *et al*., 2001).

~~In most cases,~~ Infected birds usually show no clinical signs but ~~they~~ may eventually become lethargic and emaciated. Many affected birds show diarrhoea and swollen joints, and comb and wattles may regress and become pale. Affected birds, especially gallinaceous poultry, are usually older than 1 year. Some show respiratory signs ~~and~~, including sudden death may occur, dyspnoea is less common, and granulomatous ocular lesions (Pocknell *et al*., 1996) ~~as well as~~ and skin lesions have been reported. Under intensive husbandry conditions, sudden death may occur, often associated with severe lesions in the liver; such lesions are easily observed at post-mortem examination (Salamatian *et al.,* 2020; Tell *et al*., 2001).

The primary lesions of avian tuberculosis in ~~birds~~ poultry (chickens and turkeys) are nearly always in the intestinal tract. Such lesions take the form of deep ulcers filled with caseous material containing many mycobacterial cells, and these are discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall~~, but~~. Still, when the intestine is opened, the true nature of the mass becomes evident. Typical caseous lesions are nearly always found in the liver and spleen~~, and~~; these organs are usually ~~are~~ greatly enlarged because of the formation of new tuberculous tissue. The lungs and other tissues are ordinarily free from lesions even in advanced cases (Salamatian *et al.,* 2020; Tell *et al.*, 2001; Thorel *et al.*, 1997).

~~Among domestic animals (mammals), domestic pigs (~~*~~Sus scrofa~~* ~~f.~~ *~~domesticus~~*~~) are the most susceptible to avian tuberculosis. Usually, no clinical manifestations are observed in such animals. Avian tuberculosis is suspected when tuberculous lesions are found in the head and mesenteric lymph nodes on meat inspection after slaughter. Findings of tuberculous lesions that involve other organs (liver, spleen, lungs, etc.) are rare, usually occurring at the advanced stage of the disease.~~ *~~Mycobacterium a. avium~~* ~~accounted for up to 35% of the~~ *~~Mycobacteria~~* ~~isolated from such tuberculous lesions (Dvorska~~ *~~et al.,~~* ~~1999; Pavlik~~ *~~et al.,~~* ~~2003, 2005; Shitaye~~ *~~et al.,~~* ~~2006). Unlike the other species mentioned previously, cattle are highly resistant to the causative agent of avian tuberculosis, and tuberculous lesions are detected in head lymph nodes, or occasionally in liver lymph nodes, only on meat inspection.~~ *~~Mycobacterium a. avium~~* ~~can be successfully isolated from tuberculous lesions in mesenteric lymph nodes from juvenile cattle: the isolation rate from cattle under 2 years of age was 34.4% in contrast to 13.0% from cattle over 2 years of age (Dvorska~~ *~~et al.,~~* ~~2004).~~

Pet and wild birds with avian mycobacteriosis have clinical presentations often exacerbated by age and viral and fungal co-infections (Schmidt *et al.,* 2022; Schmitz *et al.,* 2018b). The presence of nonspecific clinical signs and the absence of gross finds during necropsy in psittacine and passeriform birds may confound diagnosis. Furthermore, differences in body condition and gross pathology are observed, where psittacines have more severe lesions than passeriform birds. These differences could also be attributed to the fact that they are often more likely infected with *M. genavense* than *M*. *avium* (Schmitz *et al.,* 2018a). The advent of more affordable WGS has allowed the study of *M*. *avium* and *M*. *genavense* and their epidemiology in a large captive population of birds belonging to multiple taxa for over 22 years. In this large bird population, 68% of all birds at necropsy had isolates that were infected with *M. avium* or *M. genavense*. The WGS study of these mycobacterium isolates demonstrated strong evidence of disease clustering among those birds infected with *M. avium* but not among those harbouring *M. genavense* (Witte *et al.,* 2021). This works sheds light on the epidemiology of mycobacterium among captive birds, and future studies are necessary to understand these pathogens’ epidemiology better and to help identify its reservoirs.

It is essential to bear in mind that all members of *M. avium* ~~complex~~ and *M. genavense* are capable of giving rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (Narsana *et al.,* 2023; Pavlik *et al.*, 2000; Tell *et al*., 2001). ~~Members of~~ *~~Mycobacterium avium~~* ~~complex are classed in Risk Group 2 for human infection and should be handled with appropriate measures~~ All *Mycobacterium* species can cause infection in people (Cowman *et al.,* 2019). Caution should be exercised by those working with birds in environments infected with *Mycobacterium*, especially those immunosuppressed. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by conducting a thorough risk assessment as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. ~~Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4.~~ The CDC’s online Manual for Biosafety in Microbiological and Biomedical Laboratories is also a good reference[[2]](#footnote-3).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian tuberculosis 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 and identification of the agent(a) |
| Ziehl–Neelsen staining | – | – | – | ++ | – | – |
| Culture | – | – | – | ++ | – | – |
| ~~Haemagglutination (stained antigen)~~ | ~~+~~ | ~~+++~~ | ~~+~~ | ~~–~~ | ~~++~~ | ~~–~~ |
| PCR  | +++ | – ~~+~~ | ++ ~~–~~ | +++ | + ~~–~~ | – |
| Detection of immune response |
| Haemagglutination (stained antigen) | + | +++ | + | – | ++ | – |
| Tuberculin test | ++ | +++ | + | – | ++ | – |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
(a)A combination of agent identification methods applied to the same clinical sample is recommended.

1. Identification of the agent

If there is a characteristic history of avian tuberculosis in a flock and typical lesions are found in birds at post-mortem, the detection of acid-fast bacilli (AFB) in smears or sections from affected organs, stained by the Ziehl–Neelsen method usually is ~~normally~~ sufficient to establish a diagnosis. Confirmation of *M. avium* subspecies should be carried out by PCR or other molecular techniques (Kaevska *et al.,* 2010; Slana *et al.,* 2010). Occasionally a case will occur, presumably ~~as a result of~~ due to large infecting doses giving rise to acute overwhelming disease, in which affected organs, most obviously the liver, have a ‘morocco leather’ appearance with fine greyish or yellowish mottling. ~~In such cases~~ AFB may not be found in such cases, but careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol-fuchsin stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed AFB, with unusually high resistance to penetration of the stain. ~~Recently,~~ DNA probes ~~and~~, polymerase chain reaction (PCR), and WGS techniques have been used to identify the agent at the species and subspecies level specifically. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry *(*MALDI-TOF MS*)* is a valuable tool as well (Fernández-Esgueva *et al.,* 2021). Traditionally, *M. a. avium* is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C (*M. a. avium*). The method has limited value, as other species are able to grow at 42°C. *Mycobacterium genavense* is particularly fastidious and has ~~special~~ unique requirements for growth and identification (Shitaye *et al.,* 2010).

1.1. Culture

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. The liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with the culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali, or acid to eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian tuberculosis* [*infection with* Mycobacterium tuberculosis *complex*]). *Mycobacterium a. avium* grows best on media such as Lowenstein–Jensen, Herrold’s medium, Middlebrook 7H10 ~~and~~, 7H11, or Coletsos, with 1% sodium pyruvate added. It ~~may occasionally be~~ is necessary to incorporate mycobactin J, as it is used ~~for the isolation of~~ to isolate *M. ~~a~~*~~.~~*~~paratuberculosis~~ genavense* and *M. a. silvaticum.* ~~Growth may be confined to the edge of the condensation water.~~ Cultures should be incubated for at least ~~8~~ 12 weeks, less if using liquid media. Typically, *M. a*.*avium* produces ‘smooth’ colonies within 2–4 weeks; rough variants ~~do~~ occur. Shorter incubation times can be achieved using the liquid culture ~~BACTEC~~ system or the automated fluorescent ~~MGIT 960~~ culture system. *Mycobacterium a. avium* can also be detected in ~~massively~~ infected tissue by a conventional PCR, which also ~~allows acceleration of the~~ accelerates pathogen detection and identification (Moravkova *et al.,* 2008). ~~Currently,~~ Direct detection and quantification of *M. a. avium* using IS*901* quantitative real-time PCR can be considered ~~as~~ the ~~best~~ fast and inexpensive method ~~(despite its rather high cost per test)~~ (Kaevska *et al.,* 2010; Slana *et al.,* 2010).

For *M. genavense*, the optimal medium is liquid media supplemented with Mycobactin J (an iron chelator) and then plated onto a solid medium ~~is~~ such as Middlebrook 7H11 ~~medium acidified to pH 6 and supplemented with blood and charcoal (Realini~~ *~~et al~~*~~., 1999)~~. The incubation period at 37°C with 5–7% CO2 should be extended for at least ~~6 months~~ 42 days. If samples are directly plated onto solid media, plates should be held for at least 12 weeks. Bacterial growth should be prepared in a smear and stained using an acid-fast stain. All acid-fast organisms should be identified using MALDI-TOF (matrix assisted laser desorption ionisation–time of flight [mass spectrometry]) or PCR (Buckwalter *et al.,* 2016; Hall *et al.,* 2003; Shitaye *et al.*, 2010).

Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the ~~denomination of~~ *M. avium* complex denomination. Seroagglutination, ~~which is~~ based on the sugar residue specificity of surface glycopeptidolipids, allows ~~classification~~ the parsing of *M. avium* complex organisms into 28 serovars (Wolinsky & Schaefer, 1973). More sophisticated typing methods directed at cell-wall-specific targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, ~~and~~ high-performance liquid chromatography ~~(HPLC)~~, and WGS. Based on DNA–rRNA hybridisation serovars 1 to 6, 8 to 11, and 21 ~~are currently~~ have been ascribed to *M. a. avium* and *M. a. hominissuis*, and serovars 7, 12 to 20, and 25 to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be serotyped (Inderlied *et al*., 1993). For final species and subspecies identification, the current methods are WGS and bioinformatic analysis of isolates obtained from sick birds. Avian tuberculosis in birds is commonly caused by *M. a. avium* types 1, 2, or 3. If the isolate is not one of these three serotypes, further molecular identification tests ~~(IS~~*~~901~~* ~~PCR)~~ must be ~~carried out~~ conducted in a specialised laboratory. However, it should be ~~borne in mind~~ noted that ~~superficial~~ tuberculous lesions in ~~caged pet~~ captive birds, especially psittacines, may be caused by *M. tuberculosis,* and ~~IS~~*~~6110~~* ~~PCR should be used for~~ precise identification should always be attempted (Hoop, 2002; Lanteri *et al.*, 2011; Peters *et al.,* 2007; Schmidt *et al.,* 2008; Tell *et al.,* 2001).

1.2. Nucleic acid recognition methods

Specific and reliable genetic tests for speciation ~~are currently~~ have been available (Saito *et al*., 1990)~~.~~ , including commercial nucleic ~~acid~~ hybridisation probes ~~have become a ‘gold standard’~~ reference method for ~~distinction between~~ distinguishing *M. avium,* ~~and~~ *M. intracellulare* ~~cultures.~~ and *M. genavense* ~~can also be distinguished with these tests~~. A further probe that covers the whole *M. avium* complex was also developed, as genuine *M. avium* complex strains have been described that fail to react with specific *M. avium* and *M. intracellular* probes (Soini *et al*., 1996). Nevertheless, identification errors were reported due to the cross-reactivity, which may have serious consequences (van Ingen *et al.,* 2009). Various in-house molecular methods have been reported ~~for the identification of~~ to identify mycobacterial cultures, including ~~MAC.~~ members of the *Mycobacterium avium* complex. The following gene segments could be used to identify *Mycobacterium* isolates as *M. avium* in one multiplex PCR reaction: IS*900*, IS*901*, IS*1245*. The isolates of *M. a. avium/M. a. silvaticum* are IS*900−*, IS*901+*, IS*1245+*, the isolates of *M. a. hominissuis* are IS*900−*, IS*901−*, IS1*245+*, and the isolates of *M. a. paratuberculosis* are IS*900+*, IS*901−*, IS*1245−* (Kaevska *et al.,* 2010; Moravkova *et al.,* 2008). A ~~multiplex~~ 16S rRNA PCR and sequencing method for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex ~~has some advantages (Cousins~~ *~~et al~~*~~., 1996). 16S rRNA~~ is currently commercially available. Similarly, many veterinary diagnostic laboratories commonly perform in-house PCR and sequencing (Kirschner *et al*., 1993) ~~may also be used~~. Culture-independent in-house molecular tests have been developed ~~for the detection~~ to detect and ~~identification of~~ identify species belonging to the *M. avium* complex directly from samples (Hall *et al.,* 2003; Kaevska *et al.,* 2010). WGS of isolates has recently become the go-to molecular method to identify mycobacterium isolates from birds with great accuracy. It enables, with the use of bioinformatic tools, not only an accurate identification of species and subspecies, but also helps to determine the organism relatedness within a flock or environment (Witte *et al.,* 2021). In recent years, veterinary diagnostic laboratories have extensively adopted real-time PCR methods to detect *M. a. avium* directly from different specimens (faeces, tissues, formalin-fixed tissues, and environmental samples). The technique rapidly detects fastidious and slow-growing microorganisms, such as *M. a. avium* (Tell *et al.,* 2003a; 2003b).

Several commercial diagnostic PCR tests for detecting *M. a. avium* are available. Still, users should consider the skill set and equipment necessary to perform such tests. Furthermore, it is important to determine the fitness for the purpose of these tests before implementation. The interpretation of the results of these molecular tests also requires veterinary expertise.

*~~Mycobacterium a. avium,~~* ~~the causative agent of avian tuberculosis (Thorel~~ *~~et al.,~~* ~~1990), previously designated as~~ *~~M. avium~~* ~~species only, is assigned to serotypes 1 to 3 within the~~ *~~M. avium~~* ~~complex of 28 serotypes (Wolinsky & Schaefer, 1973). As revealed by molecular biological studies, the detected insertion sequence IS~~*~~901~~* ~~(Kunze~~ *~~et al.,~~* ~~1992) is possessed not only by the isolates of the above-named serotypes, but also by isolates, virulent for birds, that could not be typed because agglutination occurred (Pavlik~~ *~~et al.,~~* ~~2000). In epidemiological studies, a standardised IS~~*~~901~~* ~~RFLP methods replaced serotyping (Dvorska~~ *~~et al.,~~* ~~2003).~~

2. Immunological methods

Tests used for export depend on the importing requirement of individual countries. In the main, the tuberculin test or the haemagglutination (stained antigen) test are most frequently used for export testing of poultry.

2.1. Tuberculin test

The tuberculin test is the most widely used test ~~in~~ for domestic fowl and the only test for which an international standard for the reagent exists. Tuberculin is the standard avian purified protein derivative (PPD). Birds are tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately 2000 International Units [IU]), using a ~~very~~ fine needle of approximately 26 gauge, 10 mm long ~~× 0.5 mm~~. The test is read after 48 hours ~~and~~. A positive reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross oedema extending into the other wattle and down the neck. ~~With practice,~~ Even very small wattles on immature birds can be inoculated successfully. However, ~~in immature birds~~ the comb may be used in immature birds, although the results are not ~~so~~ as reliable. Tuberculin testing of the wattle in turkeys is much less ~~reliable~~ consistent than in ~~the~~ domestic ~~fowl~~ chickens. Inoculation in the wing web has been recommended as ~~being~~ more efficient, but this is still not as good as ~~for domestic fowl~~ in chickens. Other birds may also be tested in the wing web, but results are not generally satisfactory. The bare ornamental skin areas on Muscovy ducks and some ~~species of~~ pheasant species can be used, but ~~reliability~~ dependability is doubtful, and interpretation is difficult. Testing in the foot web of waterfowl has also been described; the test is not very sensitive and is often complicated by infections of the inoculation site.

In the common pheasant (*Phasianus colchicus*), the tuberculin test can be performed in either of two ways. In the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive result is indicated by marked swelling at the injection site after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the thoracic muscles, and the birds are observed for 6–10 hours. Infected birds will show signs of depression and keep aside from the flock, and there may be cases of sudden death. No clinical signs will be provoked in uninfected birds.

2.2. Stained antigen test

The stained-antigen agglutination test has been used with good results, especially in domestic and ornamental waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for agglutination. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may be most clearly seen as an accumulation of the malachite-green-stained antigen around the edge of the drop, leaving the centre a normal blood-red colour. This test is especially useful for screening large flocks for immediate culling and therefore has advantages over the tuberculin test for controlling the disease, even in domestic fowl. It has also been claimed that it is more reliable in domestic poultry than the tuberculin test.

2.2.1. Preparation of the antigen

An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test (Rozanska, 1965). The strain used to prepare the stained antigen must be smooth and not auto-agglutinate in saline suspension. It must conform to the characteristics of *M. a. avium*, preferably obtained from a culture collection, to guarantee its authenticity.

A strain that will detect infection with any serotype is recommended instead of the specific serotype most likely to be encountered (in Europe, serotype 2 for domestic fowl, serotype 1 for waterfowl, and birds and swine in the USA). Using a highly specific strain for the serotype is recommended. The specificity of strains can be determined only by testing them as antigens, although, in general, a serotype 2 antigen will always detect serotype 3 infection and vice versa. Serotype 1 strains detect a wide spectrum of infections and frequently detect infections with mycobactin-dependent mycobacteria or *M. a. silvaticum*. There is no reason not to use a culture containing more than one strain of *M. a. avium* if it shows the desired properties of sensitivity and specificity. Consistency of results between batches will be easier using pure cultures.

The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1% sodium pyruvate. Good growth should be obtained in approximately 7 days. The liquid culture is used as a seed for bulk antigen preparation.

Antigen for agglutination tests is best obtained on a solid medium, such as Löwenstein–Jensen or 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. Using a solid medium maximizes the chance of detecting contamination, and antigens grown in some liquid media are not agglutinated by specific antibodies. Liquid seed culture should be diluted (based on experience) to give discrete colonies on the solid medium. This will usually give the best yield increasing the chance of detecting contamination. About 10 ml of inoculum will usually allow it to wash over the whole surface and provide sufficient moisture to keep the air in the bottle near 100% humidity.

The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains. The antigen is harvested by adding sterile glass beads and twice the volume of sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash off all the growth, and the washing is collected into a sterile bottle and re-incubated at 37°C for 7 days. The killed bacilli are washed twice in sterile normal saline with 0.2% formalin by centrifugation and re-suspension. This sequence is safer than the original method in which the washing was carried out before the incubation that kills the organisms. Finally, bacilli are again centrifuged and re-suspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate to a concentration of about 1010 bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland’s scale.

Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The suspension made from the culture washings is also re-examined microscopically (for likely contaminants such as yeasts) and rechecked by culture to ensure that the formalin has killed the mycobacteria.

2.2.2. Validation of the antigen

Cultures should be checked by Gram staining for contamination by organisms other than mycobacteria.

One or more batches for agglutinating antigen must be tested for efficacy in using serum from naturally or artificially infected tuberculous birds by comparison with a standard preparation of known potency. When using animals for research or reagent testing, approval of the procedures and the use of animals by the institution’s ethics committee should be sought before any testing occurs. The potency relative to that of the standard preparation must not differ significantly from that declared on the label. Each bottle of antigen must be tested with normal chicken serum (to detect autoagglutination) and *M. a. avium* positive chicken serum of low and high antibody content. This should be done, where possible, alongside a previous batch of stained antigens. Those bottles that give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained. This is done by adding 3 ml of 1% malachite green solution per 100 ml of suspension. The stained antigen should be checked using whole blood, just as the unstained antigen was tested with serum. The agglutinating antigen should stay in the refrigerator for at least 6 months at 4°C and much longer if frozen at –20°C or below. If a batch has not been used for several weeks, it should be rechecked, especially for autoagglutination.

It is critical to perform a safety test of the unwashed antigen by culture and incubation to ensure that all the bacilli are dead.

Note on limitation of use

Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any value in cases of *M. tuberculosis* infection in ~~caged~~ pet birds.

C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS

1. Background

No vaccines are available.

Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated products of growth of *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity ~~as a means of identifying~~ to identify birds infected with or sensitised to the same species of ~~tubercle bacillus~~ *Mycobacterium*. Importantly it is also used ~~as an~~ to aid ~~to~~ differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis (see Chapter 3.1.13). An international standard preparation of PPD-A is being developed by WOAH to replace the former WHO Standard[[3]](#footnote-4).

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

2. Outline of production and minimum requirements for tuberculin production

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Strains of *M. a. avium* used to prepare seed cultures should be purchased from a culture collection and identified as ~~to~~ species by appropriate tests. Several strains are recommended ~~by~~ for this purpose in different countries. For example, in the European Union ~~(EU), for example, are~~, D4ER and TB56~~. Reference may also be made to~~ are recommended. The relevant national recommendations should be followed. Globally there are commercial sources for PPD-A.

2.1.2. 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 ~~with~~ of sufficient potency. The necessary tests are described below.

2.2. Method of manufacture

2.2.1. 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 ~~[TCA]~~ 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, ~~which are~~ then sealed to prevent contamination. The product may be freeze-dried.

2.2.2. Requirements for ingredients

The production culture substrate must be shown to ~~be capable of producing~~ produce a product that conforms to the ~~standards of the~~ European Pharmacopoeia (~~2000~~ 2024[[4]](#footnote-5)) standards or other international standards such the WHO (WHO, 1987). It must be free from ingredients known to cause toxic or allergic reactions.

2.2.3. 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 ~~is determined by the Kjeldahl method~~. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

2.2.4. Final product batch tests

i) Sterility

Sterility testing is generally performed according to the European Pharmacopoeia (~~2000~~ 2024) or other guidelines (see ~~also~~ 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 C.2.2.4.iv. ~~In guinea-pigs sensitised with~~ *~~M. bovis~~*~~,~~ 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 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. ~~The following is the previously described method, using experimental animals to evaluate the safety of PPD. The use of animals for this purpose should be reviewed and approved by the institution’s ethics committee. Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously treated 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 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 ~~carried out~~ conducted according to the ~~specifications of the~~ European Pharmacopoeia (~~2000~~ 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 ~~each of~~ three occasions with the equivalent of 500 ~~IU~~ 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 µgs of PPD. Each guinea-pig, together with ~~each of~~ 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 ~~the same~~ 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*~~, by comparison~~ 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 ~~to each, by deep intramuscular injection,~~ 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 ~~as follows: Shave~~. Briefly, have the guinea-pigs’ flanks shaved (an area large enough ~~so as~~ 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 ~~according to~~ 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 ~~of the reactions~~ 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~~s~~. 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 ~~of~~ 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. Requirements for authorisation/registration/licensing

3.1. Manufacturing process

The manufacturing process should follow the requirements of European Pharmacopoeia (~~2000~~ 2024) or other international standards.

3.2. Safety requirements

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~~s~~ 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.

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

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 (~~but~~ not exceeding 25°C) and protected from ~~the~~ light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

~~Provided the tuberculins are~~ 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 following periods ~~subsequent to~~ after the last satisfactory potency test: Liquid PPD tuberculin~~s~~: 2 years; lyophilised PPD-A tuberculin~~s~~: 8 years; HCSM (heat-concentrated synthetic-medium) tuberculin~~s~~ diluted: 2 years. 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 is currently (2024) no WOAH Reference Laboratory for avian tuberculosis
(please consult the WOAH Web site for the current list:
<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>[http://www.oie.int/](http://www.oie.int)).

**NB:** First adopted in 1989 as Tuberculosis in birds. Most recent updates adopted in 2014.

1. <https://lpsn.dsmz.de/species/mycobacterium-avium> [↑](#footnote-ref-2)
2. https://www.cdc.gov/labs/pdf/SF\_\_19\_308133-A\_BMBL6\_00-BOOK-WEB-final-3.pdf [↑](#footnote-ref-3)
3. PPD of *M. avium* tuberculin, WHO (1955) Technical Report Series, no.96, 11. [↑](#footnote-ref-4)
4. https://www.edqm.eu/en/d/234640?p\_l\_back\_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative [↑](#footnote-ref-5)