**Annex 9. Item 5.1. – Chapter 3.3.6. Avian tuberculosis**

2 MEETING OF THE WOAH BIOLOGICAL STANDARDS COMMISSION

3 **Paris, 4–8 September 2023**

4

5 **CHAP T E R 3 . 3 . 6 .**

# 6 A V I A N T U B ER C U L OS I S

7 **SUMMARY**

1. ***Description of the disease:*** *Avian tuberculosis, or avian mycobacteriosis, is ~~an important~~ a significant*
2. *disease that affects companion, captive exotic, wild, and domestic birds and mammals. The disease is most*
3. *often caused by* Mycobacterium avium *subsp.* avium (M. a. avium), a member of the M. avium complex.
4. *However, more than ten other mycobacterial species have been reported to infect birds. The most significant*
5. *cause of poultry disease is M*. *a*. *avium.*
6. *Clinical signs of the disease vary depending on the organs involved. The classical presentation is*
7. *characterised by chronic and progressive wasting and weakness. Diarrhoea is common and joint swelling*
8. *are standard features in infected flocks. Some birds may show respiratory signs, and occasionally, sudden*
9. *death occurs. Some birds may develop granulomatous ocular lesions.*
10. Mycobacterium tuberculosis*, the agent that most commonly causes human tuberculosis* (*gene IS*61101) *is*
11. *less commonly rarely the cause of infection in birds, and it is often ~~as a~~ the result of transmission from pet*
12. *bird owners.*
13. *Members of* M. avium *complex:* M. a. avium (*serotypes 1–3; containing gene segments IS*901 *and IS*1245),
14. M. avium *subsp.* hominissuis (*serotypes 4–6, 8–11, and 21; lacking gene segment IS*901 *and containing*
15. *segment IS*1245) *and* M. intracellulare (*serotypes 7, 12–20, and 22–28; lacking both IS*901 *and IS*1245) *can*
16. *also infect an extensive range of mammals such as swine, cattle, deer, sheep, goats, horses, cats, dogs,*
17. *and exotic species. In humans, all members of the* M. avium *complex and* M. genavense *~~are capable of~~*
18. *inducing can induce a progressive disease that is refractory to treatment, ~~mostly~~ mainly in*
19. *immunocompromised patients.*
20. *~~All manipulations involving~~ Due to the contagious nature of this group of organisms, handling ~~of~~ open live*
21. *cultures or ~~of~~ material from infected birds must only be carried out ~~with~~ after an appropriate ~~biorisk~~*
22. *~~management~~ risk assessment and the implementation of biosafety measures designed to avoid infection.*
23. *Diagnosis of avian tuberculosis in birds depends on the demonstration of* ~~the above-mentioned~~ a
24. mycobacterial species *in live or dead birds or the detection of an immune response, cellular or humoral,*
25. *culture examination, or gene segments ~~IS~~*~~6110~~*~~, IS~~*~~901~~ *~~and IS~~*~~1245~~ *by polymerase chain reaction* (*PCR*) *in*
26. *the excretions or secretions of live birds.*
27. ***Detection of the agent:*** *Where clinical signs of avian tuberculosis are seen in the flock, or typical*
28. *tuberculous lesions are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or*
29. *sections made from affected organs is sufficient for a quick positive diagnosis. If acid-fast bacilli are not*
30. *found but typical tuberculous signs or lesions are present in the birds, a culture of the organism or PCR must*
31. *be attempted~~. PCR could also be carried out directly~~ on tissue samples. Any acid-fast organism isolated*
32. *should be identified by nucleic-acid-based tests or chromatographical* (*e.g. high-performance liquid*
33. *chromatography* [*HPLC*]) *criteria; serotyping of isolates of* M. avium *complex members or PCR for 16S* r*RNA*
34. g*ene followed by sequencing, or the presence of an amplicon for the insertion sequences IS*6110*, IS*901,
35. *and IS*1245 *could also be performed.*
36. ***Tuberculin ~~test~~ and serological tests:*** *These tests are ~~normally~~ typically used to determine ~~the~~ disease*
37. *prevalence ~~of disease~~ in a flock or ~~to~~ detect infected birds. When used to detect the presence of avian*
38. *tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.*
39. *In domestic fowl, the tuberculin test in the wattle is the test of choice. This test is less useful in other species*
40. *of bird. ~~A better test, especially in waterfowl, is~~ The whole blood stained-antigen agglutination test is better,*
41. *especially in waterfowl. It is more reliable and ~~has the advantage that it will~~ can give a result within a few*
42. *minutes while the bird is still being held.*
43. ***Requirements for vaccines and diagnostic biologicals:*** *No vaccines are available for use in birds. Avian*
44. *tuberculin purified protein derivative* ~~(~~*~~PPD~~*~~)~~ *is the standard preparation for use in the tuberculin test of*
45. *domestic poultry. Avian PPD is also used as a component in the comparative intradermal tuberculin test in*
46. *cattle* (*see Chapter 3.1.13* Mammalian tuberculosis [infection with *Mycobacterium tuberculosis* complex])*.*

## 54 A. INTRODUCTION

1. Several mycobacterial species can be involved in the aetiology of avian tuberculosis ~~and~~, also known as avian
2. mycobacteriosis. Avian tuberculosis is most commonly ~~produced~~ caused by infection with *Mycobacterium avium* subsp.
3. *avium* (serotypes 1, 2, and 3: containing specific gene segment IS*901* and nonspecific segment IS*1245*) and less frequently
4. by *M. genavense* (Guerrero *et al.*, 1995; Pavlik *et al.*, 2000; Salamatian *et al.,* 2020; Sattar *et al.,* 2021; Tell *et al*., 2001).
5. Avian mycobacteriosis is also caused by other two members of the *M. avium* complex: *M. avium* subsp. *hominissuis*
6. (serotypes 4–6, 8–11, and 21: lacking gene segment IS*901* and containing segment IS*1245* and mainly infecting humans
7. and pigs) and *M. intracellulare* (serotypes 7, 12–20, and 22–28: lacking both gene segments IS*901* and IS*1245*) and by
8. *~~M. intracellulare~~*~~,~~ *~~M. scrofulaceum~~*~~,~~ *~~M. fortuitum,~~* ~~and~~ other potentially pathogenic mycobacterial species including
9. *M. scrofulaceum* and *M. fortuitum*. Under some circumstances, an extensive range of mammalian species, such as swine,
10. cattle, deer, sheep, goats, horses, cats, dogs, and exotic animals, can be infected by these mycobacterium species
11. (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).
12. *Mycobacterium tuberculosis* and *M. bovis* are ~~less common as causal~~ rarely the causative agents of tuberculosis in birds
13. (Hoop, 2002; Lanteri *et al.,* 2011; Peters *et al.,* 2007; Schmidt *et al.,* 2022; Tell *et al*., 2001).
14. *Mycobacterium avium* species with standing in nomenclature as of 2023[35](#_bookmark113) (Arahal *et al.,* 2023) consists of ~~four~~ three
15. subspecies: *M. avium* subsp. *avium*, *~~M. avium~~* ~~subsp.~~ *~~hominissuis~~*~~,~~ *M. avium* subsp. *silvaticum*, and *M. avium* subsp.
16. *paratuberculosis* (Mijs *et al.*, 2002; Thorel *et al*., 1990). The latter is the causal agent of Johne’s disease, or
17. paratuberculosis, in ruminants and other mammalian species (see Chapter 3.1.16 *Paratuberculosis* [*Johne’s disease*]).
18. *Mycobacterium a. silvaticum,* which like *M. avium* subsp. *paratuberculosis* grows *in-vitro* only on media with Mycobactin,
19. which can cause avian tuberculosis in wood pigeons (Thorel *et al.*, 1990). With the widespread use of whole genome
20. sequencing (WGS) and bioinformatics, some studies have investigated the classification of species belonging to the genus
21. *Mycobacterium* and have proposed that *M. avium* be three subspecies *M. avium* subsp. *avium*, *M. avium* subsp.
22. *paratuberculosis*, and *M. avium* subsp. *lepraemurium*. Further subdividing *M. avium* subsp. *avium* into three variants *M.*
23. *avium* subsp. *avium* var. *avium*, *M. avium* subsp. *avium* var. *silvaticum*, and *M. avium* subsp. *avium* var. *hominissuis* (Riojas
24. *et al.,* 2021; Tortoli *et al.,* 2019).
25. ~~All~~ *~~M. a avium~~* ~~isolates from birds and mammals, including humans, have a multiple repetitive sequence IS~~*~~901~~* ~~in their~~
26. ~~genome and produce a characteristic three-band pattern in IS~~*~~1245~~* ~~restriction fragment length polymorphism (RFLP) as~~
27. ~~described and standardised previously (Dvorska~~ *~~et al.~~*~~, 2003; Ritacco~~ *~~et al~~*~~., 1998). This repetitive sequence is also present~~
28. in *M. a. silvaticum* and RFLP analysis can help with identification. IS*901* has only been detected in *M. avium* strains with
29. ~~serotypes 1, 2 and 3 (Pavlik~~ *~~et al~~*~~., 2000; Ritacco~~ *~~et al~~*~~., 1998) that are apparently more pathogenic to birds than other~~
30. ~~serotypes (Tell~~ *~~et al~~*~~., 2001). On the basis of genetic and phenotypic differences it has recently been proposed to~~
31. ~~differentiate~~ *~~M. a. avium~~* ~~into two subspecies based on the target organism:~~ *~~M. a. hominissuis~~* ~~for human and porcine~~
32. isolates and *M. a. avium* for bird-type isolates (Mijs *et al*., 2002). *Mycobacterium a. hominissuis* has polymorphic multiband
33. ~~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~~
34. ~~noting that the typical features of bird-isolates, the three-band pattern in IS~~*~~1245~~* ~~RFLP and presence of IS~~*~~901~~*~~, have also~~
35. ~~been found in cervine and bovine isolates of~~ *~~M. a. avium~~*~~.~~
36. Avian tuberculosis in birds is most prevalent in gallinaceous poultry and in wild birds raised in captivity. Turkeys are ~~quite~~
37. susceptible, but ducks, geese, and other water birds are comparatively resistant. The practices of allowing poultry to roam
38. at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of the causal
39. agent of avian tuberculosis among them. Infected individuals and contaminated environments (water and soil) are the ~~main~~
40. primary sources of infection. The above-mentioned mycobacterial species causing avian tuberculosis can survive for
41. several months in the environment (Dvorska *et al.*, 2007; Kazda *et al.*, 2009; Shitaye *et al.*, 2008; Tell *et al*., 2001).
42. <https://lpsn.dsmz.de/species/mycobacterium-avium>
43. ~~In most cases,~~ Infected birds usually show no clinical signs but ~~they~~ may eventually become lethargic and emaciated.
44. Many affected birds show diarrhoea and swollen joints, and comb and wattles may regress and become pale. Affected
45. birds, especially gallinaceous poultry, are usually older than 1 year. Some show respiratory signs ~~and~~, including sudden
46. death may occur, dyspnoea is less common, and granulomatous ocular lesions (Pocknell *et al*., 1996) ~~as well as~~ and skin
47. lesions have been reported. Under intensive husbandry conditions, sudden death may occur, often associated with severe
48. lesions in the liver; such lesions are easily observed at post-mortem examination (Salamatian *et al.,* 2020; Tell *et al*., 2001).
49. The primary lesions of avian tuberculosis in ~~birds~~ poultry (chickens and turkeys) are nearly always in the intestinal tract.
50. Such lesions take the form of deep ulcers filled with caseous material containing many mycobacterial cells, and these are
51. discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as
52. tumour-like masses attached to the gut wall~~, but~~. Still, when the intestine is opened, the true nature of the mass becomes
53. evident. Typical caseous lesions are nearly always found in the liver and spleen~~, and~~; these organs are usually ~~are~~ greatly
54. enlarged because of the formation of new tuberculous tissue. The lungs and other tissues are ordinarily free from lesions
55. even in advanced cases (Salamatian *et al.,* 2020; Tell *et al.*, 2001; Thorel *et al.*, 1997).
56. ~~Among domestic animals (mammals), domestic pigs (~~*~~Sus scrofa~~* ~~f.~~ *~~domesticus~~*~~) are the most susceptible to avian~~
57. ~~tuberculosis. Usually, no clinical manifestations are observed in such animals. Avian tuberculosis is suspected when~~
58. ~~tuberculous lesions are found in the head and mesenteric lymph nodes on meat inspection after slaughter. Findings of~~
59. ~~tuberculous lesions that involve other organs (liver, spleen, lungs, etc.) are rare, usually occurring at the advanced stage~~
60. ~~of the disease.~~ *~~Mycobacterium a. avium~~* ~~accounted for up to 35% of the~~ *~~Mycobacteria~~* ~~isolated from such tuberculous~~
61. lesions (Dvorska *et al.,* 1999; Pavlik *et al.,* 2003, 2005; Shitaye *et al.,* 2006). Unlike the other species mentioned previously,
62. ~~cattle are highly resistant to the causative agent of avian tuberculosis, and tuberculous lesions are detected in head lymph~~
63. ~~nodes, or occasionally in liver lymph nodes, only on meat inspection.~~ *~~Mycobacterium a. avium~~* ~~can be successfully isolated~~
64. ~~from tuberculous lesions in mesenteric lymph nodes from juvenile cattle: the isolation rate from cattle under 2 years of age~~
65. ~~was 34.4% in contrast to 13.0% from cattle over 2 years of age (Dvorska~~ *~~et al.,~~* ~~2004).~~
66. Pet and wild birds with avian mycobacteriosis have clinical presentations often exacerbated by age and viral and fungal
67. co-infections (Schmidt *et al.,* 2022; Schmitz *et al.,* 2018b). The presence of nonspecific clinical signs and the absence of
68. gross finds during necropsy in psittacine and passeriform birds may confound diagnosis. Furthermore, differences in body
69. condition and gross pathology are observed, where psittacines have more severe lesions than passeriform birds. These
70. differences could also be attributed to the fact that they are often more likely infected with *M. genavense* than *M*. *avium*
71. (Schmitz *et al.,* 2018a). The advent of more affordable WGS has allowed the study of *M*. *avium* and *M*. *genavense* and
72. their epidemiology in a large captive population of birds belonging to multiple taxa for over 22 years. In this large bird
73. population, 68% of all birds at necropsy had isolates that were infected with *M. avium* or *M. genavense*. The WGS study
74. of these mycobacterium isolates demonstrated strong evidence of disease clustering among those birds infected with *M.*
75. *avium* but not among those harbouring *M. genavense* (Witte *et al.,* 2021). This works sheds light on the epidemiology of
76. mycobacterium among captive birds, and future studies are necessary to understand these pathogens’ epidemiology better
77. and to help identify its reservoirs.
78. It is essential to bear in mind that all members of *M. avium* ~~complex~~ and *M. genavense* are capable of giving rise to a
79. progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (Narsana *et*
80. *al.,* 2023; Pavlik *et al.*, 2000; Tell *et al*., 2001). ~~Members of~~ *~~Mycobacterium avium~~* ~~complex are classed in Risk Group 2 for~~
81. ~~human infection and should be handled with appropriate measures~~ All *Mycobacterium* species can cause infection in
82. people (Cowman *et al.,* 2019). Caution should be exercised by those working with birds in environments infected with
83. *Mycobacterium*, especially those immunosuppressed. All laboratory manipulations with live cultures or potentially
84. infected/contaminated material must be performed at an appropriate biosafety and containment level determined by
85. conducting a thorough risk assessment as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing*
86. *biological risk in the veterinary laboratory and animal facilities*. ~~Biocontainment measures should be determined by risk~~
87. ~~analysis as described in Chapter 1.1.4.~~ The CDC’s online Manual for Biosafety in Microbiological and Biomedical
88. Laboratories is also a good reference [36](#_bookmark114).

1. [https://www.cdc.gov/labs/pdf/SF 19\_308133-A\_BMBL6\_00-BOOK-WEB-final-3.pdf](https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf)

## 142 B. DIAGNOSTIC TECHNIQUES

143 ***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** | ++ | +++ | + | – | ++ | – |

144 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

145 + = suitable in very limited circumstances; – = not appropriate for this purpose.

1. PCR = polymerase chain reaction.
2. (a)A combination of agent identification methods applied to the same clinical sample is recommended.

#### 1. Identification of the agent

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

##### 1.1. Culture

1. If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are seen in smears
2. or sections, an attempt must be made to isolate the causative organism from the necropsy material. The liver or
3. spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory
4. as it could be less contaminated. As with the culture of *M. bovis*, non-sterile specimens need to be processed with
5. detergent, alkali, or acid to eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian*
6. *tuberculosis* [*infection with* Mycobacterium tuberculosis *complex*]). *Mycobacterium a. avium* grows best on media
7. such as Lowenstein–Jensen, Herrold’s medium, Middlebrook 7H10 ~~and~~, 7H11, or Coletsos, with 1% sodium pyruvate
8. added. It ~~may occasionally be~~ is necessary to incorporate mycobactin J, as it is used ~~for the isolation of~~ to isolate
9. *M. ~~a~~*~~.~~ *~~paratuberculosis~~ genavense* and *M. a. silvaticum.* ~~Growth may be confined to the edge of the condensation~~
10. ~~water.~~ Cultures should be incubated for at least ~~8~~ 12 weeks, less if using liquid media. Typically, *M. a*. *avium* produces
11. ‘smooth’ colonies within 2–4 weeks; rough variants ~~do~~ occur. Shorter incubation times can be achieved using the
12. liquid culture BACTEC system or the automated fluorescent MGIT 960 culture system. *Mycobacterium a. avium* can
13. also be detected in ~~massively~~ infected tissue by a conventional PCR, which also ~~allows acceleration of the~~ accelerates
14. pathogen detection and identification (Moravkova *et al.,* 2008). ~~Currently,~~ Direct detection and quantification of
15. *M. a. avium* using IS*901* quantitative real-time PCR can be considered ~~as~~ the ~~best~~ fast and inexpensive method
16. ~~(despite its rather high cost per test)~~ (Kaevska *et al.,* 2010; Slana *et al.,* 2010).
17. For *M. genavense*, the optimal medium is liquid media supplemented with Mycobactin J (an iron chelator) and then
18. plated onto a solid medium ~~is~~ such as Middlebrook 7H11 ~~medium acidified to pH 6 and supplemented with blood and~~
19. ~~charcoal (Realini~~ *~~et al~~*~~., 1999)~~. The incubation period at 37°C with 5–7% CO2 should be extended for at least ~~6 months~~
20. 42 days. If samples are directly plated onto solid media, plates should be held for at least 12 weeks. Bacterial growth
21. should be prepared in a smear and stained using an acid-fast stain. All acid-fast organisms should be identified using
22. MALDI-TOF (matrix assisted laser desorption ionisation–time of flight [mass spectrometry]) or PCR (Buckwalter *et*
23. *al.,* 2016; Hall *et al.,* 2003; Shitaye *et al.*, 2010).
24. Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional
25. biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*.
26. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the ~~denomination~~
27. ~~of~~ *M. avium* complex denomination. Seroagglutination, ~~which is~~ based on the sugar residue specificity of surface
28. glycopeptidolipids, allows ~~classification~~ the parsing of *M. avium* complex organisms into 28 serovars (Wolinsky &
29. Schaefer, 1973). More sophisticated typing methods directed at cell-wall-specific targets are currently available, such
30. as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, ~~and~~ high-performance liquid
31. chromatography ~~(HPLC)~~, and WGS. Based on DNA–rRNA hybridisation serovars 1 to 6, 8 to 11, and 21 ~~are currently~~
32. have been ascribed to *M. a. avium* and *M. a. hominissuis*, and serovars 7, 12 to 20, and 25 to *M. intracellulare*.
33. However, no consensus was achieved on other serovars, and some isolates cannot be serotyped (Inderlied *et al*.,
34. 1993). For final species and subspecies identification, the current methods are WGS and bioinformatic analysis of
35. isolates obtained from sick birds. Avian tuberculosis in birds is commonly caused by *M. a. avium* types 1, 2, or 3. If
36. the isolate is not one of these three serotypes, further molecular identification tests ~~(IS~~*~~901~~* ~~PCR)~~ must be ~~carried out~~
37. conducted in a specialised laboratory. However, it should be ~~borne in mind~~ noted that ~~superficial~~ tuberculous lesions
38. in ~~caged~~ pet birds, especially psittacines, may be caused by *M. tuberculosis,* and ~~IS~~*~~6110~~* ~~PCR should be used for~~
39. precise identification should always be attempted (Hoop, 2002; Lanteri *et al.*, 2011; Peters *et al.,* 2007; Schmidt *et*
40. *al.,* 2008; Tell *et al.,* 2001).

##### 1.2. Nucleic acid recognition methods

1. Specific and reliable genetic tests for speciation ~~are currently~~ have been available (Saito *et al*., 1990). , including
2. commercial nucleic ~~acid~~ hybridisation probes ~~have become a ‘gold standard’~~ reference method for ~~distinction between~~
3. distinguishing *M. avium,* ~~and~~ *M. intracellulare* ~~cultures.~~ and *M. genavense* ~~can also be distinguished with these tests~~.
4. A further probe that covers the whole *M. avium* complex was also developed, as genuine *M. avium* complex strains
5. have been described that fail to react with specific *M. avium* and *M. intracellular* probes (Soini *et al*., 1996).
6. Nevertheless, identification errors were reported due to the cross-reactivity, which may have serious consequences
7. (van Ingen *et al.,* 2009). Various in-house molecular methods have been reported ~~for the identification of~~ to identify
8. mycobacterial cultures, including ~~MAC.~~ members of the *Mycobacterium avium* complex. The following gene segments
9. could be used to identify *Mycobacterium* isolates as *M. avium* in one multiplex PCR reaction: IS*900*, IS*901*, IS*1245*.
10. The isolates of *M. a. avium/M. a. silvaticum* are IS900−, IS901+, IS1245+, the isolates of *M. a. hominissuis* are
11. IS900−, IS901−, IS1245+, and the isolates of *M. a. paratuberculosis* are IS900+, IS901−, IS1245− (Kaevska *et al.,*
12. 2010; Moravkova *et al.,* 2008). A ~~multiplex~~ 16S rRNA PCR and sequencing method for differentiating *M. avium* from
13. *M. intracellulare* and *M. tuberculosis* complex ~~has some advantages (Cousins~~ *~~et al~~*~~., 1996). 16S rRNA~~ is currently
14. commercially available. Similarly, many veterinary diagnostic laboratories commonly perform in-house PCR and
15. sequencing (Kirschner *et al*., 1993) ~~may also be used~~. Culture-independent in-house molecular tests have been
16. developed ~~for the detection~~ to detect and identification of identify species belonging to the *M. avium* complex directly
17. from samples (Hall *et al.,* 2003; Kaevska *et al.,* 2010). WGS of isolates has recently become the go-to molecular
18. method to identify mycobacterium isolates from birds with great accuracy. It enables, with the use of bioinformatic
19. tools, not only an accurate identification of species and subspecies, but also helps to determine the organism
20. relatedness within a flock or environment (Witte *et al.,* 2021). In recent years, veterinary diagnostic laboratories have
21. extensively adopted real-time PCR methods to detect *M. a. avium* directly from different specimens (faeces, tissues,
22. formalin-fixed tissues, and environmental samples). The technique rapidly detects fastidious and slow-growing
23. microorganisms, such as *M. a. avium* (Tell *et al.,* 2003a; 2003b).
24. Several commercial diagnostic PCR tests for detecting *M. a. avium* are available. Still, users should consider the skill
25. set and equipment necessary to perform such tests. Furthermore, it is important to determine the fitness for the
26. purpose of these tests before implementation. The interpretation of the results of these molecular tests also requires
27. veterinary expertise.
28. *~~Mycobacterium a. avium,~~* ~~the causative agent of avian tuberculosis (Thorel~~ *~~et al.,~~* ~~1990), previously designated as~~
29. *~~M. avium~~* ~~species only, is assigned to serotypes 1 to 3 within the~~ *~~M. avium~~* ~~complex of 28 serotypes (Wolinsky &~~
30. ~~Schaefer, 1973). As revealed by molecular biological studies, the detected insertion sequence IS~~*~~901~~* ~~(Kunze~~ *~~et al.,~~*
31. ~~1992) is possessed not only by the isolates of the above-named serotypes, but also by isolates, virulent for birds, that~~
32. ~~could not be typed because agglutination occurred (Pavlik~~ *~~et al.,~~* ~~2000). In epidemiological studies, a standardised~~
33. ~~IS~~*~~901~~* ~~RFLP methods replaced serotyping (Dvorska~~ *~~et al.,~~* ~~2003).~~

#### 2. Immunological methods

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

##### 2.1. Tuberculin test

1. The tuberculin test is the most widely used test ~~in~~ for domestic fowl and the only test for which an international
2. standard for the reagent exists. Tuberculin is the standard avian purified protein derivative (PPD). Birds are tested by
3. intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately 2000 International
4. Units [IU]), using a ~~very~~ fine needle of approximately 26 gauge, 10 mm long ~~× 0.5 mm~~. The test is read after 48 hours
5. ~~and~~. A positive reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross
6. oedema extending into the other wattle and down the neck. ~~With practice,~~ Even very small wattles on immature birds
7. can be inoculated successfully. However, ~~in immature birds~~ the comb may be used in immature birds, although the
8. results are not ~~so~~ as reliable. Tuberculin testing of the wattle in turkeys is much less ~~reliable~~ consistent than in ~~the~~
9. domestic ~~fowl~~ chickens. Inoculation in the wing web has been recommended as ~~being~~ more efficient, but this is still
10. not as good as ~~for domestic fowl~~ in chickens. Other birds may also be tested in the wing web, but results are not
11. generally satisfactory. The bare ornamental skin areas on Muscovy ducks and some ~~species of~~ pheasant species
12. can be used, but ~~reliability~~ dependability is doubtful, and interpretation is difficult. Testing in the foot web of waterfowl
13. has also been described; the test is not very sensitive and is often complicated by infections of the inoculation site.
14. In the common pheasant, the tuberculin test can be performed in either of two ways. In the first, 0.05 ml or 0.1 ml of
15. tuberculin is injected into the skin of the lower eyelid. A positive result is indicated by marked swelling at the injection
16. site after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the thoracic muscles, and the birds are observed
17. for 6–10 hours. Infected birds will show signs of depression and keep aside from the flock, and there may be cases
18. of sudden death. No clinical signs will be provoked in uninfected birds.

##### 2.2. Stained antigen test

1. The stained-antigen agglutination test has been used with good results, especially in domestic and ornamental
2. waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by
3. venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for agglutination.
4. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may be most clearly seen
5. as an accumulation of the malachite-green-stained antigen around the edge of the drop, leaving the centre a normal
6. blood-red colour. This test is especially useful for screening large flocks for immediate culling and therefore has
7. advantages over the tuberculin test for controlling the disease, even in domestic fowl. It has also been claimed that it
8. is more reliable in domestic poultry than the tuberculin test.
9. 2.2.1. Preparation of the antigen
10. An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test
11. (Rozanska, 1965). The strain used to prepare the stained antigen must be smooth and not auto-
12. agglutinate in saline suspension. It must conform to the characteristics of *M. a. avium*, preferably
13. obtained from a culture collection, to guarantee its authenticity.
14. A strain that will detect infection with any serotype is recommended instead of the specific serotype
15. most likely to be encountered (in Europe, serotype 2 for domestic fowl, serotype 1 for waterfowl, and
16. birds and swine in the USA). Using a highly specific strain for the serotype is recommended. The
17. specificity of strains can be determined only by testing them as antigens, although, in general, a
18. serotype 2 antigen will always detect serotype 3 infection and vice versa. Serotype 1 strains detect a
19. wide spectrum of infections and frequently detect infections with mycobactin-dependent
20. mycobacteria or *M. a. silvaticum*. There is no reason not to use a culture containing more than one
21. strain of *M. a. avium* if it shows the desired properties of sensitivity and specificity. Consistency of
22. results between batches will be easier using pure cultures.
23. The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1%
24. sodium pyruvate. Good growth should be obtained in approximately 7 days. The liquid culture is used
25. as a seed for bulk antigen preparation.
26. Antigen for agglutination tests is best obtained on a solid medium, such as Löwenstein–Jensen or
27. 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. Using
28. a solid medium maximizes the chance of detecting contamination, and antigens grown in some liquid
29. media are not agglutinated by specific antibodies. Liquid seed culture should be diluted (based on
30. experience) to give discrete colonies on the solid medium. This will usually give the best yield
31. increasing the chance of detecting contamination. About 10 ml of inoculum will usually allow it to
32. wash over the whole surface and provide sufficient moisture to keep the air in the bottle near 100%
33. humidity.
34. The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most
35. strains. The antigen is harvested by adding sterile glass beads and twice the volume of sterile normal
36. saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently
37. to wash off all the growth, and the washing is collected into a sterile bottle and re-incubated at 37°C
38. for 7 days. The killed bacilli are washed twice in sterile normal saline with 0.2% formalin by
39. centrifugation and re-suspension. This sequence is safer than the original method in which the
40. washing was carried out before the incubation that kills the organisms. Finally, bacilli are again
41. centrifuged and re-suspended in sterile normal saline containing 0.2% formalin and 0.4% sodium
42. citrate to a concentration of about 1010 bacteria per ml. This corresponds to a concentration ten times
43. that which matches tube No. 4 on McFarland’s scale.
44. Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The
45. suspension made from the culture washings is also re-examined microscopically (for likely
46. contaminants such as yeasts) and rechecked by culture to ensure that the formalin has killed the
47. mycobacteria.
48. 2.2.2. Validation of the antigen
49. Cultures should be checked by Gram staining for contamination by organisms other than
50. mycobacteria.
51. One or more batches for agglutinating antigen must be tested for efficacy in using serum from
52. naturally or artificially infected tuberculous birds by comparison with a standard preparation of known
53. potency. When using animals for research or reagent testing, approval of the procedures and the use
54. of animals by the institution’s ethics committee should be sought before any testing occurs. The
55. potency relative to that of the standard preparation must not differ significantly from that declared on
56. the label. Each bottle of antigen must be tested with normal chicken serum (to detect
57. autoagglutination) and *M. a. avium* positive chicken serum of low and high antibody content. This
58. should be done, where possible, alongside a previous batch of stained antigens. Those bottles that
59. give satisfactory agglutination reactions with the antisera can now be pooled and the antigen stained.
60. This is done by adding 3 ml of 1% malachite green solution per 100 ml of suspension. The stained
61. antigen should be checked using whole blood, just as the unstained antigen was tested with serum.
62. The agglutinating antigen should stay in the refrigerator for at least 6 months at 4°C and much longer
63. if frozen at –20°C or below. If a batch has not been used for several weeks, it should be rechecked,
64. especially for autoagglutination.
65. It is critical to perform a safety test of the unwashed antigen by culture and incubation to ensure that
66. all the bacilli are dead.

#### Note on limitation of use

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

## 328 C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS

#### 1. Background

1. No vaccines are available.
2. Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated products of growth of
3. *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity ~~as a means of identifying~~ to identify birds
4. infected with or sensitised to the same species of ~~tubercle bacillus~~ *Mycobacterium*. Importantly it is also used ~~as an~~ to aid
5. ~~to~~ differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis (see Chapter 3.1.13). An
6. international standard preparation of PPD-A is being developed by WOAH to replace the former WHO Standard [37](#_bookmark115).
7. The general principles ~~as given~~ in Chapter 1.1.8 *Principles of veterinary vaccine production,* should be followed for
8. injectable diagnostic biologicals such as tuberculin. The standards ~~set out~~ here and in chapter 1.1.8 are intended to be
9. 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

1. 2.1.1. Biological characteristics of the master seed
2. Strains of *M. a. avium* used to prepare seed cultures should be purchased from a culture collection
3. and identified as ~~to~~ species by appropriate tests. Several strains are recommended ~~by~~ for this
4. purpose in different countries. For example, in the European Union ~~(EU), for example, are~~, D4ER
5. and TB56~~. Reference may also be made to~~ are recommended. The relevant national
6. recommendations should be followed. Globally there are commercial sources for PPD-A.
7. 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)
8. Seed cultures should be shown to be free from contaminating organisms and to be capable of
9. producing tuberculin ~~with~~ of sufficient potency. The necessary tests are described below.

##### 2.2. Method of manufacture

1. 2.2.1. Procedure
2. The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid
3. media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily
4. accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid’s
5. medium). When the culture has been adapted to a liquid medium, it can be maintained by a passage
6. at 2–4-week intervals (Angus, 1978; Haagsma & Angus, 1995).
7. The organism is cultivated in modified Dorset-Henley’s synthetic medium, then killed by heating in
8. flowing steam and filtered to remove cells. The protein in the filtrate is precipitated chemically
9. (ammonium sulphate or trichloroacetic acid ~~[TCA]~~ are used), washed, and resuspended. An
10. antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more
11. than 0.5% [w/v]), may be added. Mercurial derivatives should not be used. Glycerol (not more than
12. 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically
13. into sterile neutral glass containers, ~~which are~~ then sealed to prevent contamination. The product
14. may be freeze-dried.
15. 2.2.2. Requirements for ingredients
16. The production culture substrate must be shown to ~~be capable of producing~~ produce a product that
17. conforms to the ~~standards of the~~ European Pharmacopoeia (~~2000~~ 2024 [38](#_bookmark116) ) standards or other
18. international standards such the WHO (WHO, 1987). It must be free from ingredients known to cause
19. toxic or allergic reactions.
20. 2.2.3. In-process controls
21. The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time
22. period. Any flasks showing contamination or grossly abnormal growth should be discarded after
23. autoclaving. As incubation proceeds, the surface growth of many cultures becomes moist and may
24. sink into the medium or to the bottom of the flask. In PPD-A tuberculin, the pH of the dissolved
25. precipitate (the so-called concentrated tuberculin) should be pH 6.6–6.7. The Kjeldahl method
26. determines the protein level (total organic nitrogen) of the PPD-A concentrate ~~is determined by the~~
27. ~~Kjeldahl method~~. Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.
28. 2.2.4. Final product batch tests
29. PPD of *M. avium* tuberculin, WHO (1955) Technical Report Series, no.96, 11.

1. <https://www.edqm.eu/en/d/234640?p_l_back_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative>
2. i) Sterility
3. Sterility testing is generally performed according to the European Pharmacopoeia (~~2000~~ 2024) or
4. other guidelines (see ~~also~~ Chapter 1.1.9 *Tests for sterility and freedom from contamination of*
5. *biological materials intended for veterinary use*).
6. ii) Identity
7. One or more batches of tuberculin may be tested for specificity together with a standard preparation
8. of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis*
9. using a procedure similar to that described in Section C.2.2.4.iv. ~~In guinea-pigs sensitised with~~
10. *~~M. bovis~~*~~,~~ The potency of the preparation of avian tuberculin must be shown to be not more than 10%
11. of the potency of the standard preparation of bovine tuberculin used in the potency test. The use of
12. animals for this purpose should be reviewed and approved by your institution's ethical committee.
13. iii) Safety
14. Tuberculin PPD-A can be examined for freedom from living mycobacteria using the culture method
15. described previously. This culture method, which does not require the use of animals, is used in many
16. laboratories, and its use is encouraged over the use of animals for this purpose. The following is the
17. previously described method, using experimental animals to evaluate the safety of PPD. The use of
18. animals for this purpose should be reviewed and approved by the institution’s ethics committee. Two
19. guinea-pigs, each weighing not less than 250 g and ~~that have~~ not ~~been treated~~ previously treated
20. with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the
21. tuberculin under test. No abnormal effects should occur within 7 days.
22. Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately
23. before it is dispensed into final containers or on samples taken from the final containers themselves.
24. A sample of at least 10 ml must be taken and ~~this must be~~ injected intraperitoneally or subcutaneously
25. into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is
26. desirable to take a larger sample, 50 ml, and to concentrate any residual mycobacteria by
27. centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and are
28. examined macroscopically at post-mortem. Any lesions found are examined microscopically and by
29. culture. Each filled container must be inspected before it is labelled, and any showing abnormalities
30. must be discarded.
31. A test for the absence of toxic or irritant properties must be ~~carried out~~ conducted according to the
32. ~~specifications of the~~ European Pharmacopoeia (~~2000~~ 2024) specifications or the equivalent
33. regulatory documents for each country or region.
34. To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any
35. material that could interfere with the test are each injected intradermally on ~~each of~~ three occasions
36. with the equivalent of 500 ~~IU~~ International units – one IU is equal to the biological activity 0.02 µg of
37. PPD – of the preparation under test in a 0.1 ml volume. In the USA and Canada, the potency of the
38. tuberculin is expressed as tuberculin unit (TU) rather than IU. One TU is also defined as 0.02 µgs of
39. PPD. Each guinea-pig, together with ~~each of~~ the three control guinea-pigs that have not been injected
40. previously, is injected intradermally 15–21 days after the third injection with the same dose of ~~the~~
41. ~~same~~ tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different
42. when measured 24–28 hours later.
43. iv) Batch potency
44. The potency of avian tuberculin is determined in guinea-pigs sensitised with *M. a. avium*~~, by~~
45. ~~comparison~~ compared with a standard preparation calibrated in IU or TU.
46. Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by
47. administering ~~to each, by deep intramuscular injection,~~ a suitable dose of inactivated or live
48. *M. a. avium* to each by deep intramuscular injection. The test is performed between 4 and 6 weeks
49. later ~~as follows: Shave~~. Briefly, have the guinea-pigs’ flanks ~~so as~~ to provide space for three-to-four
50. injections on each side. Prepare at least three dilutions of the tuberculin under test and at least three
51. dilutions of the standard preparation in an isotonic buffer solution containing 0.0005% (w/v)
52. polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of
53. not less than 8 mm and not more than 25 mm. Allocate the dilutions to the injection sites randomly
54. ~~according to~~ using a Latin square design. The dilutions correspond to 0.001, 0.0002, and 0.00004
55. mg of protein in a final dose of 0.2 ml, injected intradermally.
56. At 24 hours, the reactions’ diameters ~~of the reactions~~ are measured, and the results are calculated
57. using standard statistical methods, taking the diameters to be directly proportional to the logarithms
58. of the concentrations of the tuberculin~~s~~. The estimated potency must be not less than 75% and not
59. more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of
60. error (*p* = 0.95) are not less than 50% and not more than 200% of the estimated potency. If the batch
61. fails a potency test, the test may be repeated one or more times, provided that the final estimate of
62. potency and ~~of~~ fiducial limits is based on the combined results of all the tests.
63. It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml or
64. 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

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

##### 3.2. Safety requirements

1. 3.2.1. Target and non-target animal safety
2. Antimicrobial preservatives or other substances that may be added to a tuberculin must have been shown not
3. to impair the safety and effectiveness of the product. The maximum permitted concentration~~s~~ for phenol is
4. 0.5% (w/v), and for glycerol, it is 10% (v/v). The pH should be between 6.5 and 7.5.
5. 3.2.2. Precautions (hazards)
6. Experience ~~both~~ in humans and animals led to the observation that appropriately diluted tuberculin injected
7. intradermally results in a localised reaction at the injection site without generalised manifestations. Even in
8. very sensitive persons, severe, generalised reactions are extremely rare and limited.

##### 3.3. Stability

1. During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C).
2. Freeze-dried preparations may be stored at higher temperatures (~~but~~ not exceeding 25°C) and protected from ~~the~~
3. light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.
4. ~~Provided the tuberculins are~~ Following accepted practice, tuberculin should be stored at a temperature of between
5. 2°C and 8°C and protected from light; they may be used up to the end of the following periods ~~subsequent to~~ after
6. the last satisfactory potency test: Liquid PPD tuberculin~~s~~: 2 years; lyophilised PPD-A tuberculin~~s~~: 8 years; HCSM
7. (heat-concentrated synthetic-medium) tuberculin~~s~~ diluted: 2 years. Recent research on the temperature stability of
8. human, bovine, and avian tuberculin solutions has shown that they are stable for a year at 37°C. This should be
9. further explored as these products are used in the field in remote areas of the world where maintaining temperature
10. control is very difficult (Maes *et al.,* 2011).

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612 **NB:** There is currently (2024) no WOAH Reference Laboratory for avian tuberculosis

613 (please consult the WOAH Web site for the current list:

614 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3><http://www.oie.int/>).

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