Mycobacteriosis in Wildlife: A General Approach

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Abstract

Mycobacterial species are raising serious concerns in livestock and wild animals worldwide. In wildlife, mycobacterial infection has been reported in hundreds of species and likely has the potential to occur in every vertebrate. Since this infection is of a chronic nature the best strategy to control the infection is through early identification of infected animals, and better diagnostic measures are required for effective control programs.

With the development of new molecular methods for detecting and characterizing microorganisms, the ecology of mycobacteria has rapidly advanced in all areas. In human medicine, polymerase chain reaction (PCR) assays are accepted diagnostic standards, replacing or complementing culture isolation and acid-fast staining.

The mycobacterial species that produce tuberculosis in humans and animals are included in the *Mycobacterium tuberculosis* complex (MTC). Mycobacteria from the *Mycobacterium avium* complex (MAC) cause a variety of diseases including tuberculosis-like disease in humans and birds, disseminated infections in immunocompromised patients, lymphadenitis in humans and mammals and paratuberculosis in ruminants.

This manuscript is a review of the scientific literature on the classification and biology, epidemiology, clinical signs, pathology, diagnostic techniques, and public health concerns of *Mycobacterium tuberculosis* and *Mycobacterium avium* complexes in wild mammals.

Keywords: *Mycobacterium* spp., wildlife, epidemiology, pathology, diagnostic techniques

Chapter - 1

Classification and Biology of Genus *Mycobacterium*

The *Mycobacterium* genus belongs to the family Mycobacteriaceae, the order Actinomycetales, the phylum Actinobacteria and kingdom Bacteria. The phylum Actinobacteria contains one class (Actinobacteria), five subclasses, six orders, 14 suborders, and 40 families. The orders, suborders, and families are defined based on 16S ribosomal RNA (rRNA) sequences and distinctive signature nucleotides. The suborder Corynebacterineae contains seven families with several well-known genera. Three of the most important genera are *Corynebacterium*, *Mycobacterium* (sole genus of the family Mycobacteriaceae), and *Nocardia* [1].

The species of *Mycobacterium* is composed of a group of high genomic guanine-cytosine (C+G) content (~61 to 71%), facultative intracellular, Gram-positive microorganisms comprising more than 130 established and validated species and subspecies [2], with surprisingly diverse phenotypes related to growth rate, metabolic activity, colony appearance, environmental distribution, and pathogenic potential for eukaryotic hosts [3]. Unidentified species are constantly being discovered and mycobacterial taxonomy is continuously changing. For the acceptance of a new species, the old and new ways of identification are all included: biochemical characteristics, growth and pigmentation characteristics, high performance liquid chromatography (HPLC) analysis and a unique genetic composition determined by the sequence of genes that allow species differentiation, such as the 16S rRNA gene, the *hsp65* gene and the internal transcribed spacer (ITS) ribosomal region, as applied and subsequently published in the International Journal of systematic Bacteriology Sequencing of at least two targets as mentioned above must be included, but the choice of targets is not specified [4]. Mycobacteria are acid-fast bacilli, acidophilic, small, slightly curved or straight rods that sometimes branch or form filaments. Mycobacterial filaments differ from those of actinomycetes in readily fragmenting into rods and coccoid bodies when distributed. They are aerobic, immobile, non-sporulated and catalase positive bacteria. Their cell wall is lipid-rich and contain waxes with 60 to 90 carbon mycolic acids, which are complex fatty acids with a hydroxyl group on the β-carbon and an aliphatic chain attached

to the α-carbon. The presence of mycolic acids and other lipids, in high concentration outside the peptidoglycan, makes mycobacteria acid-fast dye resistant (basic fuchsin cannot be removed from the cell by acid alcohol treatment), as well as resistant to immune system defense mechanisms and disinfectants [1, 5]. Although most of these species are saprophytic, important human and animal pathogens have been identified. Pathogenic members are usually characterized by their slow growth in culture, with generation times of 12 to 24 h, and must be incubated for 2 to 40 days after inoculation of a solidified complex medium to form a visible colony, whereas nonpathogenic members grow considerably faster [6]. The species belonging to a species group (referred to as species-complex) can be very different in virulence or pathogenesis. Several previously considered species appear to consist of several closely related species, as biochemical and mainly genetic analyses have demonstrated in, for instance, the *Mycobacterium tuberculosis* complex (MTC), considered a separate group belonging to the *Mycobacterium* genus, and a similar phenomenon can be found in the *M. avium* complex [7]. Mycobacteria other than *Mycobacterium tuberculosis* are commonly referred to as atypical or non-tuberculous mycobacteria (NTM). Two of these, *M. leprae* and *M. ulcerans* cause disease in normal hosts and are thus primary pathogenic, responsible for important diseases in humans and animals in the developed world as well as in developing countries, respectively leprosy and Buruli ulcer. They are often not regarded as NTM. The remaining species are considered environmental, saprophytes or opportunistic pathogens and cause disease when host-defenses are compromised [8]. NTM can cause a wide array of clinical diseases; pulmonary disease is most frequent, followed by lymphadenitis in children, skin disease (by *M. marinum*, particularly in fish tank fanciers), and other extrapulmonary or disseminated infections in the severely immunocompromised [9]. Non-tuberculosis mycobacteria can be arranged into four groups according to the Runyon classification: Group I consists of the photochromogenic (pigmented when exposed to light) species of slow growers (e.g., *Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium simiae*); members of group II are scotochromogenic (form pigment in the dark) slow growers (e.g., *Mycobacterium scrofulaceum, Mycobacterium szulgai, Mycobacterium gordonae*); group III contains the nonchromogenic slow growers (e.g., *Mycobacterium malmoense, Mycobacterium xenopi, Mycobacterium avium* complex, *Mycobacterium ulcerans, Mycobacterium haemophilum*); and group IV consists of rapid growers, defined as maturing in less than 1 week (e.g., *Mycobacterium fortuitum, Mycobacterium chelonae, Mycobacterium abscessus*) [10].

1.1 *Mycobacterium tuberculosis* complex (MTC)

Members of the MTC, the causative agents of tuberculosis (TB) in mammals, are highly related with remarkable nucleotide sequence homogeneity, despite varying pathogenicity, host preference, geographical range and epidemiology, and form a tight cluster in taxonomical studies [11]. This complex now consists of *Mycobacterium tuberculosis*, *Mycobacterium bovis* (including the BCG vaccine strains, that was attenuated from a clinical isolate of *M. bovis* called "lait Nocard"), *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium caprae*, *Mycobacterium canettii*, *Mycobacterium pinnipedii* and *Mycobacterium mungi* which are all pathogenic for human and/or animals and all named after their original host [11, 12]. The analysis of deletions further allowed the evolution of the *M. tuberculosis* complex to be mapped. Comparative genomic analysis showed 14 regions of difference (RD1-14), ranging in size from 2 to 12.7 kb, that were present in *M. tuberculosis*, but absent in *M. bovis* BCG [13]. The members of the *M. tuberculosis* complex had evolved from a common precursor (Figure 1). Strains that possessed TbD1 were termed "ancient" whilst those lacking the region were labelled "modern". Additionally, the assumption that *M. tuberculosis* had evolved from *M. bovis* was discredited. This finding was also confirmed by [14].

Mycobacterium tuberculosis

The complete genome of the laboratory reference strain *Mycobacterium tuberculosis* H37Rv was published in 1998 [15], and since then, genome sequences from several other *M. tuberculosis* strains have been published. *Mycobacterium tuberculosis* is the predominant cause of human TB. Although *M. tuberculosis* is considered primarily a human pathogen, an of outbreaks have been reported in other animals both domestic and wildlife species living in close contact with humans [16].

Mycobacterium bovis

Mycobacterium bovis is the major etiologic agent of animal TB, infecting many species of wild and domestic mammals and also man [17] but is primarily a bovid pathogen. Tuberculosis occurs worldwide in livestock and it's prevalence is unknown in several countries but it was estimated that over 50 million cattle could be infected [18]. Several countries have successfully eradicated bovine TB through test and removal programs reinforced by active surveillance of abattoirs. Nevertheless, in other countries (e.g., United Kingdom, Ireland, USA, New Zealand and Portugal) similar strategies could not eradicate the disease, which is now reemerging.

In Portugal, difficulties in eradicating bovine tuberculosis in cattle in some areas may relate to the occurrence of TB in wildlife reservoir species [17, 19]. Infection with *M. bovis* has been documented in a variety of wild species throughout the world (Europe, Africa, Asia, Australia, New Zealand and the Americas) in any area where livestock are raised, and in most situations these cases have been considered to be spillover from infected domestic populations and that may become maintenance hosts and reservoirs for *M. bovis* [20, 21]. *Mycobacterium bovis* infection of animals exhibits some host specificity and among farm animals, it rarely infects equidae or sheep, but occurs regularly in cattle and pigs [22]. Wildlife known to be susceptible to *M. bovis* include cervids and other artiodactylae such as wild boar (*Sus scrofa*) [19, 23]; carnivores such as coyote (*Canis latrans*) [24], wolf (*Canis lupus*) [25], gray fox (*Urocyon cinereoargentus*) [26] and red fox (*Vulpes vulpes*) [27]; insectivora (moles, voles, hedgehogs) [28]; lagomorphs [29]; rhinoceros [30]; buffaloes [31]; and primates [32]. Species such as North American bison (*Bison bison*) [33], African buffalo (*Syncerus caffer*) [34], European badgers in United Kingdom [35] and Switzerland [28], brushtail possums in New Zealand [36], white-tailed deer in Michigan [37], and several antelope species in South Africa [34] have been identified as reservoir hosts. With respect to the wild boar, its contribution to the epidemiology of TB seems variable; it is considered a spillover host in New Zealand, Australia [38] and Italy [39] but a maintenance host in Spain [40]. Moreover, wild suids were used in New Zealand as sentinels for *M. bovis* presence in the environment [41]. A wildlife reservoir species may serve as a constant source of infection for another. Studies on these wildlife reservoir hosts for *M. bovis* are providing greater insight into the epidemiology of *M. bovis* in both wildlife and domestic animal populations, which is needed to allow more effective control of the infection in domestic livestock. Once established in wildlife, TB is extremely difficult to eradicate. The only successful example is in Australia, where *M. bovis* was eradicated by depopulation of its maintenance host, the water buffalo (*Bubalus bubalis*) [42]. Therefore, before any attempt to control TB is undertaken the precise role of each species in the epidemiologic cycle needs to be ascertained [38].

Mycobacterium africanum

Mycobacterium africanum is the most common cause of pulmonary TB in people in different areas of Africa. In contrast to *M. tuberculosis* and *M. bovis*, *M. africanum* strains show a higher variability of phenotypic attributes, comprising characteristics common to both *M. tuberculosis* and *M. bovis***.** This phenotypic heterogeneity of *M. africanum* complicates its

unequivocal identification and may lead to misclassification of clinical strains. According to their biochemical characteristics, two major subgroups of *M. africanum* have been described, corresponding to their geographic origin in western (subtype I) or eastern (subtype II) Africa. Numerical analyses of these subtypes revealed that *M. africanum* subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles *M. tuberculosis* [43]. *Mycobacterium africanum* is rarely reported as a cause of TB in animals. Infection due to this variant has been described in monkeys [44], cattle and pigs in Norway [45], and in one bull in Germany [46].

Mycobacterium microti

Mycobacterium microti was associated with limited diversity and a much reduced spoligotype pattern, this group of organisms can be identified by the absence of chromosomal region $RD1^{mic.}$ [47]. This species was originally identified as a pathogen of small rodents (e.g. field voles, bank voles, wood mice) and shrews [48, 49]; although *M. microti* has been reported occasionally in a number of other species, including a badger, ferret [50], alpaca, llamas [51, 52], red deer [53], wild boar [54], squirrel monkeys [55], meerkats (*Suricata suricatta*) [56], wild boar [57], dog [58], pigs [59], domestic cats [60], and man [47, 50].

Mycobacterium canettii

Mycobacterium canettii formerly known smooth type *Mycobacterium tuberculosis* (SmTB) is the most divergent subspecies within the MTC, presenting a smooth and glossy colony morphology, a rapid grow *in vitro* and the evidence for recombination [61]. In addition *Mycobacterium canettii* strains have biochemical features such as the absence of niacin production and nitrate reduction [62] and the presence of the majority of RD, including RD9 and TbD1 [63], that differentiate them from the rest of the MTC. The first *Mycobacterium canettii* strain was isolated by Canettii in 1969 from a French farmer suffering from pulmonary TB [64] and the subspecies designation *M. canettii* was introduced by Van Soolingen et al. [65], following the isolation in 1993 in the Netherlands from a Somali child with lymphadenitis.

Mycobacterium caprae

Mycobacterium tuberculosis complex isolates recovered from goats, were originally classified as *M. tuberculosis* subsp. *caprae* [66], later this subspecies was reclassified as *M. bovis* subsp. *caprae* [67] and in 2003 was elevated to species rank as *Mycobacterium caprae* [68]. The main characteristics that differentiate isolates belonging to *M. caprae* from other

members of the MTC are a special combination of pyrazinamidase (*pncA*), catalase (*katG*) and gyrase (*gyrA*) gene polymorphisms, and specific fingerprinting patterns obtained from restriction fragment length polymorphism (RFLP) associated with insertion sequence (IS) *6110*, direct repeat and polymorphic GC-rich sequences, and direct variable repeat-spacer oligonucleotide typing (spoligotyping) that are very different to those obtained for other members [68]. *Mycobacterium caprae* has been isolated in other domesticated animals for example, cattle, pigs, sheep and rabbits [69-72] and other studies it has been described a high incidence of *M. caprae* infection in human with clinical TB acquired from bovine [73]. In wildlife *Mycobacterium caprae* has been isolated from wild boar and red deer [70, 71, 74]. In zoo animals *M. caprae* has been isolated from Siberian tiger (*Panthera tigris altaica)* in Budapest [75], dromedary camel (*Camelus dromedarius*) and bison (*Bison bison*) in Slovenia [76]. Erler et al. [77] suggested that *M. caprae* is the main cause of TB in livestock in Central European regions.

Mycobacterium pinnipedii

Tuberculosis in pinnipeds was first described for Blair in 1913 [78], but the causative agent remained unknown until 1986, when *Mycobacterium bovis,* identified biochemically, was isolated from New Zealand fur seals (*Arctocephalus forsteri*) and Australian sea lions (*Neophoca cinerea*) [79]. Later, it was discovered that the isolates from these animals differed in their genotypic characteristics and with regard to these differences, the causative agent was then thought to be a separate species among MTC members and the name "*M. pinnipedii*" was proposed, after formerly named *Mycobacterium* type seal [80]. *Mycobacterium pinnipedii* infection has been found in captive and free living pinniped species, in Australia, Argentina, France, Germany, The Netherlands, New Zealand, Uruguay and Great Britain. According to the literature, the most frequently affected pinniped species has been the Southern sea lion (*Otaria flavescens*), the majority of which kept in captivity [81].

Mycobacterium pinnipedii infection has also been described in humans (mainly zookeepers) [82, 83], one Brazilian tapir (*Tapirus terrestris*), one lama (*Lama glama*) and two lowland gorillas (*Gorilla gorilla gorilla)* from a zoo in Great Britain [80] and one Malayan tapir (*Tapirus indicus*) and a Bactrian camel (*Camelus bactrianus*) in a German zoo [84]. All of these zoological gardens had the presence of infected pinnipeds (mainly sea lion) and this is most probably the source of infection for other animals and humans. The lesions found in the animals consisted mainly of granulomas of caseous nature in the lungs and lymph nodes [80].

Mycobacterium mungi

The more recent member of MTC was identified in banded mongooses (*Mungos mungo*) that live near humans in Botswana in 2010. Host spectrum and transmission dynamics remain unknown but *Mycobacterium mungi* causes high number of deaths in banded mongooses and the time from clinical presentation to death for affected mongooses is generally short (2-3 months) compared with that for other MTC pathogens. *M. mungi* appears to infect by means of a nonrespiratory route through the nasal planum, suggestive of environmental transmission [85, 86].

1.2 *Mycobacterium avium* complex (MAC)

Originally, one species, later divided in two species, *Mycobacterium avium* and *Mycobacterium intracellulare* [87]. Recently, advances in molecular taxonomy have fuelled identified novel species within the MAC, including the *Mycobacterium chimaera* incorporating sequevar MAC-A organisms isolated from humans with pulmonary cavitations, pulmonary abscess, chronic obstructive pulmonary disease and bronchiectasis [88, 89]; the *Mycobacterium colombiense* incorporating sequevar MAC-X organisms isolated from the blood and sputum of human immunodeficiency virus (HIV) infected patients in Colombia [90], and from diseased lymph nodes in children [91, 92]; the *Mycobacterium arosiense*, recently described in an immunocompromised child with disseminated osteomyelitic lesions [93]; the *Mycobacterium vulneris* [9], *Mycobacterium marseillense*, *Mycobacterium timonense* and *Mycobacterium bouchedurhonense* isolated from patients with pulmonary disease [94]. Bacteria from the MAC differ in virulence and ecology, and are the most frequently isolated NTM [95]. *Mycobacterium* members of MAC have the capacity to survive and multiply under a wide range of environmental conditions, including low pH, extreme temperatures, chlorine or ozone treatment and low oxygen level. Thus, plus their ability to utilize many substances as nutrients, enables them to grow successfully in many biotopes [96]. The environmental sources responsible for MAC infection in different populations, the specific routes of infection and transmission, the potential for latent infection and reactivation of disease are not yet well defined [2, 3]. Ingestion of environmental organisms followed by invasion through the gastrointestinal tract has been suggested as the main route of infection because the organisms are frequently isolated from stools of different animals. There is also an important positive correlation between the presence of MAC in respiratory samples and the subsequent development of disseminated disease [97].

Several subspecies of *M. avium* have been identified based on molecular and biochemical criteria, that is HPLC of cell wall mycolic acids, sequencing of ITS ribosomal regions and RFLP of an IS*1245* [98]. These include the subsp. *avium*, subsp. *paratuberculosis*, subsp. *hominissuis* and subsp. *silvaticum* [2]. All four *Mycobacterium avium* subspecies and *Mycobacterium intracellulare* are capable of infecting a diverse range of hosts and possess a high degree of genetic similarity [7]. To date, using 16S rRNA probes, 28 MAC serotypes have been identified from which the serotypes 1-6, 8-11, and 21 belong to *Mycobacterium avium* subsp. *avium* (MAA). Serovars 7, 12-20, and 25 have been ascribed to *Mycobacterium intracellulare*. Serovar-1 is the most common organism isolated from birds and from human. Serotypes 1, 2, and 3 are considered virulent for chickens. Serotypes 1 and 2 are most commonly isolated from domestic birds, and serovar 3 is recovered sporadically from wild birds. Serotypes 1, 4, and 8 have been reported to predominate among isolates from AIDS patients [99]. Mycobacteria from the MAC cause a variety of diseases including TB-like disease in humans and birds, disseminated infections in AIDS patients and otherwise immunocompromised patients, lymphadenitis in humans and mammals and paratuberculosis in ruminants. The MAC comprises slow growing mycobacteria that are ubiquitous in the environment (soil and water), and have a wide source range, causing disease in various domestic and wild mammals and birds [96]. All ruminant species, captive or free-ranging, are susceptible to disease and death due to MAC infection [100], and a wide diversity of non-ruminant species can become infected with mycobacteria belonging to MAC.

Mycobacterium avium* subsp. *avium

Before establishing the *Mycobacterium avium* subsp. *avium* (MAA) designation, this bacterium was simply referred to as *Mycobacterium avium* and has long been recognized as a primary pathogen causing avian TB in wild and domestic birds as well as in a variety of fowl, game birds and water-fowl and has also been reported in ostriches, emus, and rheas in many zoological parks. The most common route of infection for susceptible animals is the alimentary tract. Respiratory tract is also suggested as a potential source of infection [99].

Mycobacterium avium* subsp. *paratuberculosis

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of Johne's disease or paratuberculosis, a chronic granulomatous enteritis of ruminant livestock and wildlife, with worldwide distribution

having a significant impact on the world economy [6] and has been identified in human patients with Crohn's disease [101]. For veterinary medicine, MAP is the MAC member of greatest importance, and is capable of infecting and causing disease in a wide array of animal species, including nonhuman primates, without the need for co-existent immunosuppressive infections [102]. MAP is one of the slowest growing mycobacterial species, hence primary isolation from specimen, requires prolonged culture incubation and can take several months. Unlike most other *Mycobacterium avium* subspecies, isolation of MAP requires the addition of the siderophore mycobactin to culture media [103]. From phenotypic analysis, the MAP group has been subdivided into two main types, bovine and ovine, that vary in hosts, diseases caused, and growth phenotypes [104]. Genotypically, these findings were based primarily on comparisons of the integration *loci* of the IS*900* and used polymorphisms in IS*1311* to separate sheep and cattle isolates into separate populations [105]. MAP has been isolated in a wide range of wild mammals, from deer, and in South American camelids (llamas and alpacas) [106] from rodents, badgers, raccoons, nine-banded armadillos, opossums, northern short-tailed shrew, striped skunks [107-109], wild boars [110, 111] and rhinoceros [112] to bears [113], but not all of them present the same susceptibility and develop clinical signs or lesions when infected. The close relationship between wild, captive and domestic ruminants and other species like birds is, nowadays, clinically relevant as the wild population could act as reservoir for this agent [111].

***Mycobacterium avium* subsp. *hominissuis* (MAH)**

MAC isolates of genotypes IS*901*- and IS*1245*+ and serotypes 4 to 6, 8 to 11 and 21 are less virulent for birds and are designated *M. avium* subsp. *hominissuis* (MAH). MAH was proposed to distinguish organisms found in humans and pigs from those isolated from birds [2]. Those are genomically diverse, the more diverse group of strains, low-virulence, opportunistic pathogens for both animals and humans [110]. Considered ubiquitous in the environment (the most likely source of infection for humans), MAH can cause serious systemic infection in immunocompromised patients, such as those infected with HIV. Additionally, this opportunistic pathogen can cause cervical lymphadenitis in children with cystic fibrosis, and lung infections in patients with underlying lung disease [114]. Domestic water distribution systems have been reported as possible sources of MAH infections in hospitals, family houses, and commercial places [115]. In animals, *Mycobacterium avium* subsp. *hominissuis* is found as a cause of lymphadenitis of the head and mesenteric lymph nodes of swine documented

at slaughter [102], and can also lead to systemic infection of parenchymatous organs [114]. MAH were recovered from affected lymph nodes of red deer from Austria [116].

Mycobacterium avium* subsp. *silvaticum

Mycobacterium avium subsp. *silvaticum* applies to the previously named wood pigeon bacillus, an acid-fast organism causing TB-like lesions in these wood pigeons. The inability to grow on egg media, the stimulation of growth by pyruvate, the ability to grow at pH 5.5 and their mycobactin dependency upon primary isolation, gradually losing this phenotype upon subculture, have been described as characteristics of *Mycobacterium avium* subsp. *silvaticum* [7].

Mycobacterium intracellulare

Mycobacterium intracellulare, initially named *Nocardia intracellularis*, is an environmental organism and opportunistic pathogen, isolated from a variety of animal hosts and environmental sources. *Mycobacterium intracellulare* is a closely related pathogen of birds with a low prevalence. In general, it has been subject to less study than *Mycobacterium avium*, as the latter is more prevalent in clinical and environmental samples, has a wider apparent host range, and contributes almost exclusively to disseminated MAC disease in human immunodeficiency virus patients. The type strain of *Mycobacterium intracellulare* (ATCC 13950) was isolated from humans, specifically responsible for enlarged lymph nodes in children, who died from disseminated disease [2], and progressive pulmonary disease in elderly women [117]. *Mycobacterium intracellulare* appears to have a distinct ecological niche, more prevalent in biofilms and at significantly higher colony forming units (CFU) numbers than *Mycobacterium avium* [118].

Chapter - 2

Clinical Signs, Lesions and Epidemiological Aspects in Wild Mammals

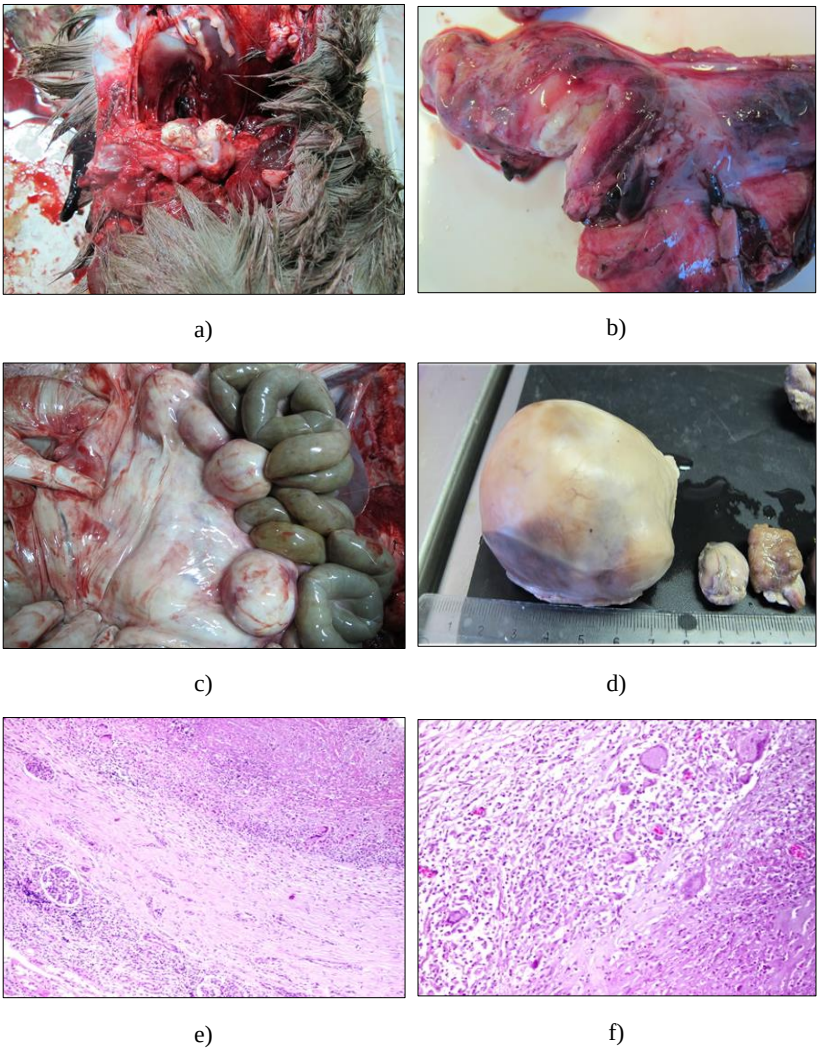
A number of significant challenges arise in detecting mycobacteriosis in free-ranging wildlife. Even in susceptible species, the majority of infected animals show no clinical signs of disease [17]. The host immune response to mycobacterial infection is complex, and significant differences exist among a diverse group of mycobacterial pathogens and host species infected. A common feature among many pathogenic mycobacterial species in both humans and animals is a prolonged asymptomatic or latent period that can last years to decades. It is interesting that during this latent period bacterial numbers are usually very low and their detection can be difficult [119].

2.1 *Mycobacterium tuberculosis* complex (MTC)

Tuberculosis caused by *Mycobacterium bovis* is a chronic, progressive disease and even in the most susceptible species, the time course of infection/disease my last for several weeks; more commonly disease will last for many months, if not years. For the majority of the course of infection, animals will be clinically normal. The most common clinical sign of disease is weight loss and this only occurs in advanced stages of the disease. Other clinical signs include swollen lymph nodes, especially of the head, discharging lymph node abscesses and signs associated with a tuberculous pneumonia such as coughing. Skeletal and synovial (elbow hygromata) lesions with associated lameness may be observed in lions [32]. Swollen head nodes with draining fistulae are almost pathognomonic in greater kudu. The severity of clinical signs may be exacerbated by environment factors such as lack of grazing during droughts. In some animal species, a change in behavior may occur in the advanced stages of disease (badger; possum; baboons) [17]. The course of TB in deer is variable [35]. The disease may be subacute or chronic, with a variable rate of progression. A small number of animals may become severely affected within a few months of infection, while others may take several years to develop clinical signs [120]. TB in deer presents as a spectrum of pathological conditions at *post mortem*, ranging from no obvious gross lesions [20] to liquefactive abscedation of lymph nodes (more typically associated with acute pyogenic bacterial infection) or

classical caseo-granulomatous lesions, closely resembling those found in cattle (proliferative granuloma, caseation, and calcification with ageing) [121]. The lesions in deer may take the form of thin-walled abscesses containing purulent material with multiple bacilli and minimal calcification or fibrosis. In cervids, TB should be considered when abscess-like lesions of unknown aetiology are observed. Thin-walled abscesses have also been observed in llamas [120]. The lymph nodes affected are usually those of the head and thorax. The mesenteric lymph nodes may be affected - large abscesses may be found at this site. The distribution of lesions will depend on the infecting dose, route of infection and the incubation period before examination [120]. TB lesions are usually found in the lymph nodes draining the nasopharynx, lung or mesenteric tissue (Figure 1 a), b) and c), respectively), most likely reflecting the different routes of transmission, either by respiratory route or by oral ingestion [122]. These lesions presented a marked heterogeneity regarding their size, ranging from very small necrotic foci to large thin-walled abscesses, sometimes exceeding 5 cm in diameter (Figure 1 d). In deer farms, the lymphoreticular tissues of the head and neck, particularly the tonsil and retropharyngeal lymph nodes were most commonly involved [123], while in wild deer, head or neck lesions are as common as mesenteric lymph nodes lesions [124]. The presence of lymphocytes and caseous necrosis were the most common features. Macrophages were also abundant, and multinucleated giant cells were observed in most of the granulomatous lesions (Figure 1 e). Langhans multinucleated giant cells and calcification were not so frequently observed when compared to the lesions in wild boars. Additionally, some of the animals presented liquefactive necrosis at the centre of the granulomatous lesions, in which cases neutrophils were also observed surrounding the necrotic debris (Figure 1 f).

These lesions are usually enclosed by a fibrous capsule. The pulmonary granulomas observed were variable in terms of dimensions as well as in their limits definition: some of them were confluent, and they all showed central necrosis involved by macrophages and other mononucleated cells.



a) b) c) d) e) f)

Fig. 1: Tuberculosis lesions in the lymph nodes of red deer (*Cervus elaphus*) with caseous necrosis. a) Retropharyngeal lymph nodes. b) Mediastinal lymph nodes. c) Mesenteric lymph nodes. d) Marked heterogeneity of Red deer abscesses containing a pale yellowish necrotic and purulent material. e) Necrotic centre of granuloma. f) Inflammatory infiltrate, composed mainly of lymphocytes, surrounding the necrotic centre and often presenting multinucleated giant cells. Hematoxylin and eosin. Original magnification: e) 100x; f) 400x.

If lesions are confined to internal lymph nodes or restricted areas of lung, the animals may not display clinical signs throughout their life. Generalized disease involving the lungs may result in emaciation. When an

infected animal shows clinical evidence of disease, death will occur within 1-2 weeks [125]. Nonspecific signs have been described, including retardation of antler growth, sexual indifference in stags in the rutting season and failure of hinds to come into estrus. Coughing and respiratory rales, although sometimes present, are not typical features of the disease in deer [125].

Lesions of TB in wild boar (*Sus scrofa*) are localized in the head lymph nodes, and TB nodules in lymph nodes seem to evolve from the necrotic to the fibronecrotic-calcified form with the "sterilization" of the nodules [126]. Nodules are never open, so that wild boars do not eliminate *M. bovis* in the environment, as happens with other wild reservoirs of infection, such as possums or badgers. Another confirmation of the self-limiting process of TB infection in wild boar lymph nodes arises from the fact that although the prevalence of lesions increases significantly with the wild boar's age, positive results upon DNA probing actually decrease with the animal's age. In the area under consideration, TB in domestic cattle was still high, and it is possible that wild boars of the area became infected by scavenging infected cattle carcasses or during rooting by ingestion of *M. bovis* present in the soil [127]. Lesions were most typically found in the retropharyngeal, submandibular lymph nodes and submandibular salivary glands; they could be moderately hypertrophied, with 1 mm miliary caseocalcareous foci, or greatly hypertrophied, with dry yellow or liquid greenish content (Figure 2 a), b) and c)). The pulmonary and mediastinal lymph nodes were rarely hypertrophied and had only some small caseocalcareous nodules. The same type of lesion was observed in the mesenteric lymph nodes only when they were incised (Figure 2 d)). Advanced induration of the lung with calcified caseum, small caseocalcareous lesion in the lung, fibrinous pleuritic; lesions in other organs or sites, such fibrinous peritonitis, miliary foci in the liver, and arthritis in leg joints may occur [128]. Microscopically, these granulomas were characterized by the presence of a partially mineralized necrotic core involved by mononucleated cells, such as macrophages and lymphocytes, and rare multinucleated giant cells, and surrounded by a well-defined fibrotic capsule (Figure 2 e) and f)). Wild boars that are reported to be infected by *M. bovis* show lesions at necropsy. Very rarely, *M. bovis* isolation occurs from wild boar carcasses with no lesions at necropsy, similarly to what is verified in other animal species (i.e. in cattle). On contrast, the presence of lesions is not sufficient to diagnose the infection correctly: diagnosis must be confirmed by a panel of laboratory tests, including bacteriological and histopathological techniques and, nowadays, with the aid of biomolecular methods.

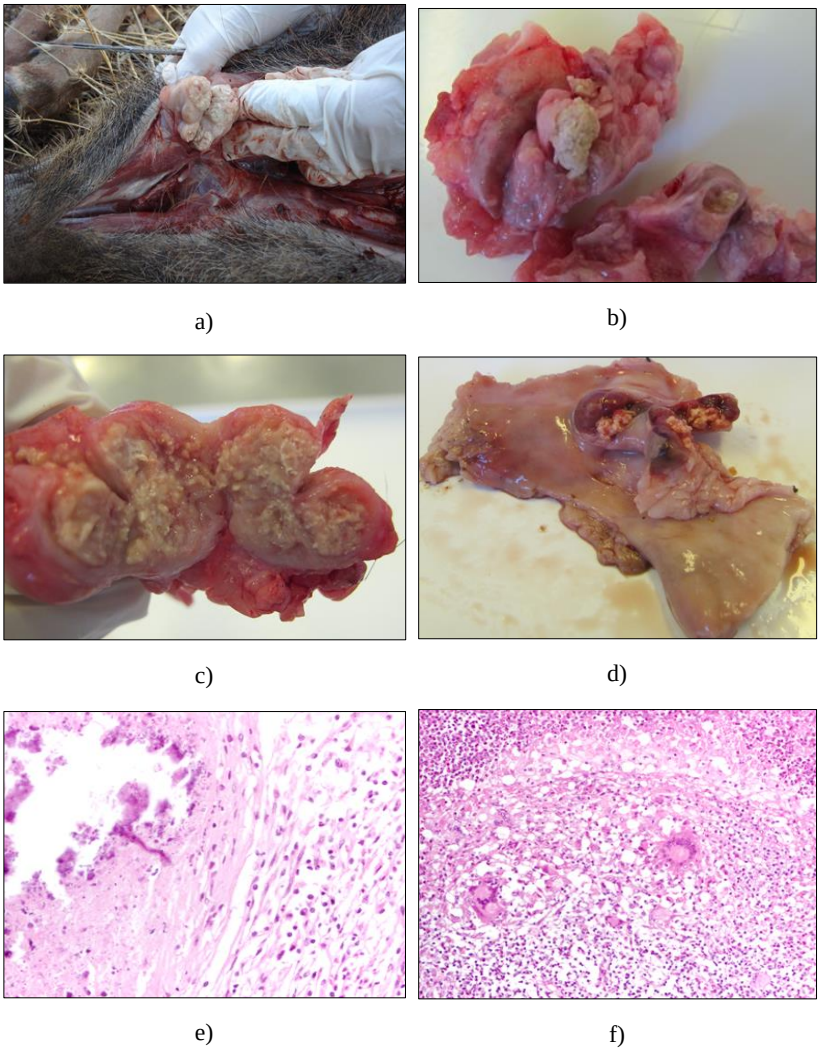


Fig 2: Tuberculosis lesions in the lymph nodes of wild boar (*Sus scrofa*) with caseocalcareous nodules. a) and b) Submandibular lymph nodes. c) Submandibular salivary gland. d) Mesenteric lymph nodes. e) Necrotic centre calcified. f) Inflammatory infiltrate, composed mainly of lymphocytes, surrounding the necrotic centre and often presenting multinucleated giant cells. Hematoxylin and eosin. Original magnification: e) 100x; f) 200x.

In tuberculous African buffalo, solid caseogranulomas are generally found in lymph nodes, whereas in the lungs the lesions vary from focal tuberculous pneumonia with pin-point areas of caseation to solid confluent caseogranulomas. Whether focal or disseminated, these lung lesions may

progress to cavitation with liquefaction [42], and such cases are extremely contagious. There have been relatively few surveys for *M. bovis* in host species other than Cervidae and Bovidae. Bruning-Fann *et al*. [26] conducted necropsies of 294 carnivores from the *M. bovis*-endemic area of Michigan and found seven animals with microscopic lesions suggestive of *M. bovis* and nine with lymph node cultures positive for *M. bovis* - six coyotes, two raccoons (*Procyon lotor*), one red fox (*Vulpes vulpes*), and one black bear (*Ursus americanus*). Moreover, gross lesions suspicious of TB were seen in two coyotes (both culture positive), and one adult male bobcat (culture negative, PCR positive). One adult male coyote had numerous 2 to 3 mm firm, white nodules that were raised above the surface of the lung, the mesenteric lymph nodes were enlarged with gritty, white mottling. Follicular hyperplasia with multiple granulomas, composed primarily of macrophages and lymphocytes, were seen histologically. Although no acid-fast bacilli were seen, *M. bovis* was cultured from these tissues. Other adult male coyote had markedly enlarged pale mesenteric lymph nodes, but only lymphoid hyperplasia was observed histologically. One adult, female bobcat had enlarged mesenteric lymph nodes (2 cm diameter) with numerous pale, tan nodules (2 mm diameter) on gross examination. Microscopic examination revealed multifocal caseogranulomas with partially mineralized necrotic debris in the centre, surrounded by lymphocytes and macrophages. Moderate numbers of acid-fast bacilli were present in the necrotic debris. No mycobacteria were isolated from these tissues. VerCauteren, et al. [24] reported culture and histopathological evidence of *M. bovis* in 58/175 (33%) free-ranging coyotes (*Canis latrans*) collected in 4-county bovine TB-endemic area in Michigan's Lower Peninsula, where cattle herds continue to be infected. Lesions occurred most commonly in the mesenteric lymph nodes (31/58), varied from focal to multifocal and ranged in size from 1 to 15 mm; however, one coyote had advanced disease with lesions occurring in the lungs and liver. A study of raccoons in Michigan, reported an estimated apparent prevalence of 2.5%, with 5/199 (Witmer et al. [129] and Palmer et al. [130] documented excretion of *M. bovis* in saliva or nasal secretions in raccoons but not in urine or faeces. Shedding of *M. bovis* has been reported for North American opossums (*Didelphis virginiana*) and experimental inoculation has demonstrated that they are relatively susceptible to *M. bovis* infection. Typical gross lesions included multifocal granulomatous pneumonia and enlarged thoracic lymph nodes and therefore, may not shed the bacteria sufficiently to serve as a reservoir for infecting livestock [131]. The ferret (*Mustela putorius*) has been regarded as highly susceptible to *M. bovis* infection, and the mesenteric lymph nodes were the most common site

of infection, with the retropharyngeal and the cervical lymph nodes also frequently involved. Gross changes examined were generally unspectacular compared with the pyogranulomatous or caseous lesions in other wild species such as possums and deer. Tuberculous lymph node lesions often included simple enlargement, which was difficult to distinguish from normal hypertrophy, occasionally darkly pigmented. Focal white nodular lesions were occasionally seen in the node parenchyma but these tuberculous lesions were grossly indistinguishable from follicular hyperplasia. Some affected nodes were oedematous, and circular cream coloured foci were apparent [132]. The lesions in affected carnivores were observed in the mesenteric lymph nodes, suggesting exposure through ingestion of scavenged material. The location of lesions, variety of species involved, and widely dispersed cases of *M. bovis* were indicative of disease spillover rather than endemic *M. bovis* in these carnivores [133]. In badgers, TB is primarily a respiratory disease resulting from inhalation of infectious aerosol particles, with dissemination to other organ systems as the infection progresses [134, 135]. Visible lesions are found most frequently in lungs, tracheobronchial and mediastinal lymph nodes, with the most common extra-thoracic sites being the head and body lymph nodes. Visible lesions are found in the abdominal cavity, but a wide range of tissues and organs are represented. Infected skin lesions are probably inflicted during social aggression or during territorial defence [135]. Although badgers are very susceptible to infection, within a population infection is most frequently seen in its latent subclinical infection form, with only a small proportion of badgers progressing to disseminated disease [135-137].

2.2 *Mycobacterium avium* complex (MAC)

Mycobacteria belonging to the MAC can affect a wide-range of wild animals, but little has been published on the clinical signs, which are rarely perceived or not documented. When present, the occurrence of clinical signs and lesions is highly variable in timing but often similar to those of their domesticated counterparts. The vast majority of reports on MAC species affecting wildlife mention the MAP and the MAA as the mycobacteria most commonly isolated in these animals. In cattle this disease is scored in four stages according to its evolution and symptoms, two of them evolving sub-clinically. Stage I, or silent infection, is the most observed in young animals, without significant clinical signs and only in *post mortem* evaluation it is possible to identify the agent by culture or histopathology. Stage II remains a subclinical disease, being observed in adult animals. It may be detected by alterations in immunological serological and/or cellular parameters.

Intermittently, fecal culture and histopathological analysis of these animals could be positive to MAP [138]. In stage III the clinical signs can be observed, occurring after several years of incubation. The initial clinical signs are subtle with gradual weight loss despite normal appetite, intermittent diarrhea along several weeks, drop in milk production and roughness of hair coat. These symptoms are included in the differential diagnosis of multiple diseases, so it is often misdiagnosed [139]. Usually, animals in this stage are positive upon enzyme-linked immunosorbent assay (ELISA) and other serological tests, as for histopathological analysis of lesions, which are common in the terminal ileum [140]. The last stage of the disease (stage IV) comprises animals that rapidly progress from the stage III with rapid condition deteriorated. They became increasingly lethargic, weak and emaciated and present intermandibular edema due to hypoproteinemia. In this stage, the culture of the agent, molecular biology techniques of PCR, ELISA, serology and histopathology, all are positive for the majority of animals tested. The gastrointestinal tract is the preferential local to sample in order to isolate the agent, but in some conditions, it can even be present in extraintestinal lesions, with the liver and lymph nodes being the most common sites [138]. The lesions observed in wild ruminants are identical to those of their domestic counterparts, while in the South American camelids the lesional pattern is similar to that of cattle. However, in llamas and alpacas, in contrast to what is generally described in cattle, lymph node necrosis and mineralization, along with multiorganic dissemination, have also been reported. As in the previously mentioned species, the most significant MAC species capable of causing clinical disease in free-living, captive and farmed deer are MAP and MAA. Although MAH has been also isolated from lesions in deer [116] and *Mycobacterium intracellulare* was also found in deer species, they are not so common and their infection is usually subclinical. Despite the occurrence of paratuberculosis in adults, outbreaks of the disease frequently occur in young deer of 8-15 months of age, contrary to the clinical disease in sheep and cattle which usually affects adults of 3-5 years of age [141]. Clinical signs of paratuberculosis in deer are similar to those described in sheep and cattle, with diarrhea and loss of weight and body condition [142]. Accordingly, the intestinal lesions of paratuberculosis in deer primarily affect the jejunum and ileum, and are identical to the typical lesions observed in sheep and goats [106]; yet, necrosis and mineralization in lymph nodes draining the gastrointestinal tract, especially those draining the ileum and ileocecal valve, are a common feature. The lymph nodes are often enlarged, and a range of changes from yellow watery areas to caseous necrosis is observed on cut surfaces. The microscopic changes in these

lesions are very similar to those caused by *Mycobacterium bovis* and other members of the MAC genus [143].

In deer with clinical signs of paratuberculosis, disseminated granulomatous lesions in the lung and liver can also be observed. A recent report in free-ranging red deer (*Cervus elaphus*) supports the possibility of multiorganic dissemination of MAP in deer, since the agent was isolated from kidneys with granulomatous lesions [144]. In deer, the infection by MAA is self-limiting as in other mammalian species [141]. The lesions may be purulent, caseous, or granulomatous, and are mainly present in the retropharyngeal lymph nodes and lymph nodes draining the intestinal tract (mesenteric and ileocecal), consistent with the feco-oral route of infection. The granulomatous lesions are grossly and microscopically identical to the lesions caused by *Mycobacterium bovis*. MAH lesions in deer are similar to those observed in animals with MAA infection, and although rare, both mycobacteria can cause systemic disease with hematogenous spread to the liver and lungs to produce miliary lesions and a terminal septicemia [121]. Despite of these findings, MAP and MAA infections can be present in apparently asymptomatic deer herds [145-146]. Furthermore, a study of wild Tule elks (*Cervus elaphus nannodes*) from California revealed no significant associations between MAC infection and microscopic lesions, such as presence of macrophages and/or multinucleate giant cells in tissue sections [146]. Significance of MAA and the clinical signs of paratuberculosis in non-ruminant wildlife are largely unknown. Lesions seem to be similar to early, subclinical infections described for ruminants and clinical signs are not systematically observed on positive animals [107]. Despite MAA being widely reported in wild boar, data on clinical infection or mortality are scarce [110]. Apparently, natural infection with this mycobateria causes barely detectable clinical signs or lesions [110]. However, there are reports of MAA isolated from free-ranging Eurasian wild boars with tuberculous lesions in intestinal lymph nodes [147], and the experimental infection with high doses of MAA results in gross and histopathological lesions of TB in tracheobronchial and mandibular lymph nodes. All visible lesions are less than 10 mm in diameter and consist of typical tuberculous granulomas with a central caseous necrosis, variably mineralized, surrounded by macrophages, lymphocytes, neutrophils, eosinophils and occasional L-MGC surrounded by fibrous tissue. Acid-fast bacilli are rarely detected in the necrotic debris of these lesions [148]. Another study also showed that in wild boars with mesenteric and submaxillar lymphadenitis, *Mycobacterium avium* subspecies type 1 and *M. avium* subspecies type 2 were the most frequently isolated mycobacteria [149].

Regarding MAH in wild boar, recent reports suggest that this animal species may act as a reservoir for these mycobacteria, since it was detected in lymph nodes without gross lesions or microscopic lesions [146, 150]. MAP was isolated from rabitts (*Oryctolagus cuniculus*), coyotes (*Canis latrans*), feral cats (*Felis familiaris*), skunks **(***Mephetis mephitis*), opossum (*Didelphis virginiana*), raccoon (*Procyon lotor*), foxes (*Vulpes vulpes*), stoats (*Mustela ermine*), weasels (*Mustela nivalis*), badgers (*Meles meles*), wood mouse (*Apodemus sylvaticus*), rats (*Rattus norvegicus*), and brown hares **(***Lepus europaeus*) [107, 151-156]. In rabbits, Greig *et al*. [155] and Maio *et al*. [156] described thickened intestines and enlarged lymph nodes in three of 33 and in two of 80 necropsied animals, respectively. Rabbits showed enlarged mesenteric lymph nodes with multiple granulomatous to abscess-like tuberculous. The core of these lesions was composed by a yellowish material varying from solid to purulent/caseous. The intestinal wall of the caecal appendix, sacculus rotundus and caecum was thickened [156]. In foxes and stoats, microscopic changes similar to those described in ruminants with subclinical paratuberculosis were noted in intestines and mesenteric lymph nodes. These changes were subtler than those seen in either advanced ruminant paratuberculosis or severely infected rabbits [107, 151, 152]. In rats, wood mice, and hares, *M. avium* subsp. *paratuberculosis* was isolated from intestinal and lymphoid tissue with few or no microscopic lesions [107]. The findings make it clear that the natural host range is far greater than previously thought and potential transmission routes to wildlife include predation, scavenging, and sympatry with infected livestock. Conversely, transmission to livestock from non-ruminant wildlife could be a factor in the epidemiology of paratuberculosis, with important implications with respect to attempted control or eradication of the disease [107].

Chapter - 3

Diagnostic Techniques

The golden standard test for *Mycobacterium* diagnosis is the microbiological culture. The culture of bacteria requires weeks or months of incubation before colony growth occurs [157]. This means that a significant amount of time is needed before a diagnosis can be made and it is also known that the sensitivity of culture is not 100%. As such, false-negative culture results may occur and it may also be difficult to isolate bacteria in culture due to intermittent shedding and a low number of bacilli [157, 158]. *Ante mortem* diagnosis is based on clinical signs, radiography and ultrasonography, leukogram, serology, intradermal tuberculin test (ITT), culture and also acid-fast staining of faecal and body fluids smear or biopsy samples from organs and tissues [159, 160] PCR is being used increasingly for primary diagnostic assays and is also playing a role in further characterisation of cultured mycobacterial strains. Its particular attraction for diagnosis is its speed; one or two days for a batch of samples compared to weeks for cultures to become positive [161]. Earlier difficulties with PCR of false negative results due to inhibition of the PCR reaction and false positive results due to cross-contamination have been largely but not completely overcome by improved extraction and contamination-control techniques. In the last few years, PCR-related techniques have continued to advance in many areas including methods of extracting DNA for amplification [162-164], as well as the implementation of real and proprietary PCR amplification systems [164]. Despite these developments, it has been found that while PCR of *Mycobacterium* spp. can provide a much faster positive diagnosis, a negative result cannot be relied on [163].

3.1 Direct smears

The fastest and simplest method of confirming a mycobacterial infection is a direct acid-fast bacilli (AFB) smear examination from tissues, faeces or other biological material. A Ziehl-Neelsen (ZN) stain or auramine stain using fluorescent microscopy can be used to determine this. Both stains work on the simple principle that any mycobacteria present in the smear retain an arylmethane dye, such as carbol fuchsin or a fluorescent analogue, within the

cell giving an acid-fast staining reaction, following treatment with a weak acid-alcohol solution. This is due to the mycolic acids in the bacterium's thick cell wall [165].

The mycobacteria, in ZN-stained smears appear as slender, often beaded, red-staining rods against a blue background, if methylene blue is the counter-stain (Figure 4a). Diagnosis of *Mycobacterium* spp. infection with this technique is poor due to low numbers of mycobacteria, these can only be visualized if at least 5 x 10^4 mycobacteria/mL of material are present and sample contamination with other acid-fast bacteria. In bovine specimens, *M. bovis* lesions are often low on mycobacteria, but *M. avium* lesions in poultry and *M. bovis* lesions in deer and badgers usually yield large numbers of mycobacteria [166]. In combination with characteristic macroscopic lesions a presumptive diagnosis of mycobacteriosis can be made, but confirmation by culture is indispensable. Direct staining does not provide any information on the species of mycobacteria causing the infection or differentiate between viable and non-viable cells [17, 165].

3.2 Isolation and identification

The critical aspects of mycobacterial culture are the procedures used for processing and decontaminating samples, the culture media employed and the methods used for identifying mycobacteria [17]. *Mycobacterium tuberculosis* and *M. avium* complex grows best in media containing eggs or egg yolk and the incubation temperature should be set at 37ºC-40ºC. Culture can be performed in Löwenstein-Jensen, Dorset´s, Herrold´s egg yolk medium, Middlebrook 7H10 and 7H11 or Coletsos medium [120, 167]. Löwenstein-Jensen supplemented with glycerol is recommended for the isolation of *M. tuberculosis*. The use of both an agar-based medium (e.g. 7H11) and an egg-based medium supplemented with 1% sodium pyruvate (e.g. Löwenstein-Jensen) is recommended for the isolation of *M. bovis*. Growth of *M. bovis* generally occurs within 3-6 weeks of incubation depending on the media used. *M. bovis* will grow on Löwenstein-Jensen medium without pyruvate, but will grow less well when sodium pyruvate is added. Cultures are incubated for a minimum of 8 weeks (preferably for 10-12 weeks) at 37°C with or without CO_2 [120]. For the isolation of MAP or *M. silvaticum* addition of mycobactin is required in all media. MAP is the slowest growing of the culturable mycobacteria [168]. Cultures should be incubated for at least 8 weeks [167]. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at weekly intervals during the incubation period [120, 167]. Mycobacteria in culture generally appear as two types of colonies: rough or smooth, and with

a shiny or opaque aspect [169]. Some species of mycobacteria, classified as photochromogens, produce carotenoid pigment in the presence of light; the group designated as scotochromogenic develops yellow colonies independent of light stimulus; the non-chromogenic group does not produce pigment; their colonies appear with shades of pale yellow or cream, in the presence of light or in darkness, and the colour does not intensify when exposed to light [170]. Typically, MAC members produce smooth and non-chromogen colonies (Figure 3 c) within 2-4 weeks, but rough variants can occur, and MTC members produce rough and non-chromogen colonies (Figure 3 d) [120, 167]. When growth is visible, smears are prepared and stained by the Ziehl-Neelsen technique (Figure 3b).

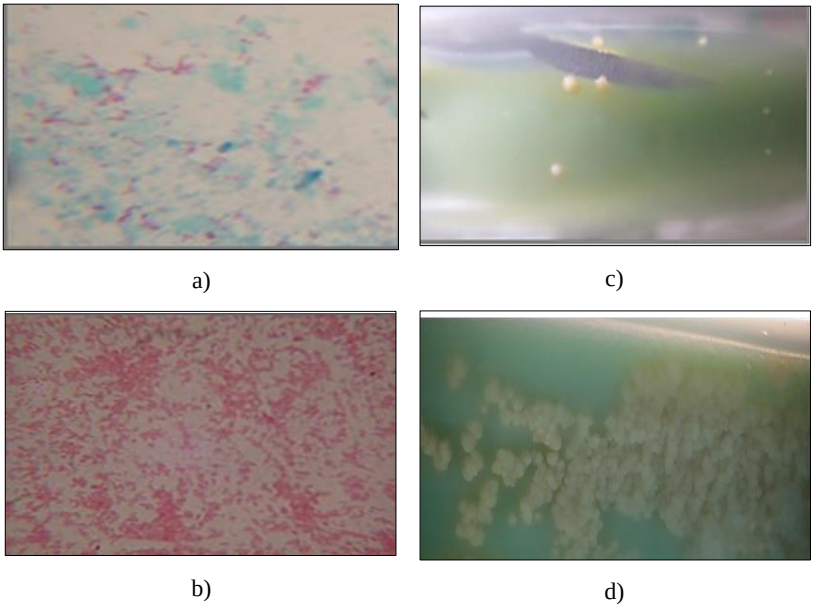


Fig 3: Ziehl-Neelsen stain and colony morphology on Löwenstein-Jensen slants. a) Acid-fast bacilli in smear examination from tissues. b) Acid-fast bacilli in smear examination from colonies. ZN stain-original magnification: 1000x. c) *Mycobacterium avium* colonies on L-J medium. d) *Mycobacterium bovis* colonies on L-J medium with pyruvate.

Identification of isolates by phenotypic characteristics of majority of clinically relevant mycobacteria can be based on growth rates, colony pigmentation and biochemical tests such as niacin production, nitrate reduction, tween 80 hydrolysis arylsulphatase, urease, tellurite reduction, thiophene-2-carboxylic acid hydrazide (TCH) sensitivity, catalase (qualitative and quantitative) growth on MacConkey and sodium chloride

tolerance [171, 172]. Although conventional biochemical assays are relatively inexpensive and simple to perform, in general, a mature growth (2-3 weeks) is required and they are often performed on a Löwenstein-Jensen (L-J) slants and require up to 3 weeks for final read of results. Furthermore, with greater than 130 mycobacterial species identified to date, the use of phenotypic methods is limited and biased to identify only the most common species of mycobacteria, underestimating the complexity of the genus and resulting in misidentification of unfamiliar species [173]. High-performance liquid chromatography (HPLC) can be used to differentiate mycobacteria based on differences in their mycolic acid profiles. Mycolic acids are high-molecular weight fatty acids with long carbon side chains present in abundance in the cell wall of mycobacteria and other organisms including *Corynebacterium*, *Rhodococcus*, and *Nocardia* species with *Mycobacterium* species containing the longest carbon chain (60-90) [174]. Although faster and more sensitive and specific compared to biochemical tests and other molecular assays, with agreement ranging from 90-99% depending on the mycobacterial species, HPLC is a technically demanding method which is not easily implemented in routine diagnostic laboratories. This method requires a high level of expertise for recognition of species based on the HPLC chromatogram [174] and is not able to differentiate between members of the MTC, except for the *M. bovis* BCG strain [175]. Rapid identification of bacterial isolates to genus can be achieved by PCR targeting genus-specific 16S rRNA in bacterial DNA extracted from the previously described culture media [173]. Non-sterile specimens need to be processed with detergent alkali or acid to eliminate rapidly growing microorganisms before culture decontamination in order to remove faster growing microbial species. Incubation with various decontamination agents such as 0.6-0.75% hexadecylpryridinium chloride (HPC) or NaOH for 3 hours to overnight, have been used. It is important that decontamination does not remove too many viable *Mycobacterium* cells [176]. Other methods like sedimentation and centrifugation can be employed if small numbers of mycobacteria are expected in the sample [177]. Shorter incubation times can be achieved using automated broth-based systems, such liquid culture. Examples of liquid culture systems are the BACTEC 460® System (Becton Dickinson Inc.), the MB/Bact® (Organon Teknika, Boxtel, Netherlands) and the BACTEC MGIT 960® (Becton Dickinson Inc.) [168]. These systems have been reported to be highly sensitive for culture [178].

3.2.1 *Mycobacterium tuberculosis* complex

The OIE [120] recommends culturing of tissue with visible lesions, such as caseous necrosis in lymph nodes (above all submandibular,

retropharyngeal, tracheobronchial, mediastinal, and mesenteric lymph nodes) and altered parenchymatous organs, e.g. lung, liver or spleen, as well as any tissues with gross lesions. When no visible lesions are detected is recommended pooled lymph node samples from head and thorax. Members of the MTC are a slow growing *Mycobacterium*, requiring in general 15 days to 40 days to grow in culture. The optimal isolation temperature for the organism is 35-37°C and the organism does not produce pigmentation even following exposure to light (non-chromogen) [179]. The most useful biochemical tests used for identification of MTC members includes: niacin accumulation, nitrate reduction, pyrazinamidase activity, inhibition of TCH, urease activity and catalase activity. Other tests that can provide additional information include tellurite reduction, Tween 80 hydrolysis, the arylsulfatase test, iron uptake and NaCl tolerance [180]. The pattern of resistance of pyrazinamide is used to distinguish between *M. tuberculosis* and *M. bovis/M. bovis* BCG strains. Growth in the presence of TCH permits the differentiation of *M. tuberculosis* that grows in the presence of the compound as opposed to other species such as *M. bovis*; it is sensitive to the compound when added to the L-J medium [180]. *M. tuberculosis* is most easily identified by nitrate reduction, and by niacin accumulation as opposed to *M. bovis* [181]. Growth in presence of P-nitrobenzoic acid also contributes to the differentiation of species of the MTC. P-nitrobenzoic acid inhibits the growth of: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* [182]. The development of biological molecular methods, in the last years, for identification of mycobacteria were implemented for clinical and research uses. Specific identification of isolates of de MTC can be made using PCR targeting single nucleotid mutations in genes *oxyR, katG, pncA, gyrA, mmpl6* and *gyrB* [80]. Moreover, the presence and/or absence of RDs can be exploited for rapid species identification [47].

3.2.2 *Mycobacterium avium* complex

Tissue culture seems to be slightly more sensitive than faecal culture and it allows the infection to be detected in some animals that had no specific lesions. Culture of MAP from faeces or tissues of other animals such as sheep and goats are less successful due to the “S” strains that usually infect these animals [168]. The best organs to use for culture are usually liver and spleen but bone marrow can be used if carcass is decomposed, as it could be less contaminated [167]. For *M. genavense* the use of BACTEC system with no additives and pH 6.0 is recommended. Middlebrook 7H11 with pH 6.0 supplemented with blood and charcoal is also recommended to promote growth of *M. genavense* [183]. In human AIDS patients, laboratory

diagnosis of MAC infection is usually made by the BACTEC blood culture [184]. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracelullare* [167]. Classification of MAC organisms have been made by seroagglutination [99]. Seroagglutination is based on sugar residue specificity of surface glycopeptidolipids, and allows classification of MAC organisms into 28 serovars: 1 to 6, 8 to 11 and 21 are currently ascribed as *M. avium*, while serovars 7, 12 to 20 and 25 to *M. intracellulare*. However, no consensus was achieved on the other serovars [167]. MAC colonies can also be identified using high HPLC for detecting mycolic acid. HPLC and the use of monoclonal antibodies to major serovars in ELISA also facilitate typing of mycobacteria [99].

3.3 Immunological methods

The diagnosis of mycobacterial infections in live wild animals remains a challenge. The intradermal tuberculin test (ITT) has proved to be a very valuable test for the diagnosis of bovine TB in cattle and has been applied in a number of different wildlife species such as in cervids, African buffalos, brushtail possums and badgers [185], but the test presents major limitations. It has been proved that ITT cannot detect some stages of infection [186]; wild ruminants must be captured twice (animals must be reexaminated 48h to 96h after injection of tuberculin) increasing the stress and the risk of accidents for the animals and for handlers [185]; the immunosuppressive effect resulting from the stress of capture as well as the effect of dehydration on skin measurement; and that intradermal testing has to be standardised for each species [17]. The tuberculin test, involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72h later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The test must be carried out on the side of the neck, with hair clipping at the site of testing, accurate intradermal injection, and careful pre- and post-inoculation skin thickness measurement using callipers to obtain results that are valid [120]. For most wildlife species, the optimal dose of PPD required for tuberculin test is unknown. The optimal dose of tuberculin is very low, in the order of 1 to 10 UI for some species such as humans, while the dose required for cattle is in the order of 3,000-5,000 UI [17]. Probes like the gamma-interferon (IFN- γ) assay and the lymphocyte proliferation assay which measure cellular immunity, and the ELISA which measures humoral immunity, could be an alternative in wild animals [185]. Advantages of IFN-γ, lymphocyte proliferation and ELISA

assays are that they employ blood and serum and they enable testing without handling the animals twice and allow repeated testing, which are important advantages in case of wild ruminants [187]. The dominant response to mycobacterial infections in ruminants is cell-mediated and the IFN-γ assay has contributed significantly to the improved detection of early *M. bovis* infection in cattle as well as an increasing number of wildlife species (e.g. non-human primates, cervids). However, a recent study demonstrated that may be of limited usefulness in some species of cervids [188]. The principle of this assay is based on the fact that when sensitised T-lymphocytes from blood of an *M. bovis*-infected animal are re-exposed to antigens from *M. bovis* (such as those in bovine PPD), the cells will release the cytokine IFN-γ. The release of IFN- γ from T-lymphocytes results in the activation of macrophages, enabling these cells to be more effective in killing intracellular pathogens such as mycobacteria. Release of IFN-γ by T-lymphocytes is regarded as a hallmark of a cellular immune response which is elicited by *M. bovis* infection [189]. Serological tests such ELISA and multiantigen print immunoassay (MAPIA) have been used to detect antimycobacterial antibodies in the serum of a range of different host species, including wildlife [190]. However, serological assays for detecting *Mycobacterium* are problematic. The sensitivity of ELISA is dependent on the stage of the disease with a higher sensitivity of the test in case of higher bacterial load. The test can detect the most severe infections in multibacillar lesions but shows lower sensitivity in animals with paucibacillar lesions [168]. One important disadvantage is the inability to distinguish between different mycobacterial infections probably due to close antigenic relationship [185].

3.4 Genetic methods

During the past several years, many molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days with a higher sensibility. Molecular biology methods offer new opportunities to differentiate, identify and type bacterial species and strains. These methods use the variability of nucleic sequences of genes such as 16S ribosomal DNA (rDNA), beta subunits of the RNA polymerase (*rpoB*) and DNA gyrase (*gyrB*), rDNA internal transcribed spacer among other genes. Some of the methods available to differentiate and identify species of mycobacteria at the DNA sequence level are PCR, PCR- restriction endonuclease analysis (REA), sequencing analysis, spoligotyping and DNA fingerprinting. These methods have been applied to both the “universal” part of the genome and to specific mycobacterial genes

[120, 167]. Isolation of mycobacterial DNA can be done from living mycobacteria, not only from mycobacterial isolates but also directly from body fluids (sputum, bronchoalveolar lavages, and bronchial and tracheal aspirates, semen, milk, blood, cerebrospinal fluid), from tissues and from faeces and can be done using dead mycobacterial cells, namely from formalin-fixed and paraffin-embedded tissues and from forensic and archaeological samples [191]. In general, a variety of methods can be used for DNA isolation from different biological materials. However, the isolation of nucleic acids from mycobacteria is more difficult than from other microorganisms because of a cell wall with thick peptidoglycan layer characteristic of the resistant to a number of lysis buffers. One of the most common mechanical disruption methods, and several suggested to lyse mycobacteria, is the bead beating, a general term for using small beads mixed with the sample, usually in the presence of a proteolytic enzyme and lysis buffer, to break tissues or tough cell walls and spores by forceful shaking in a cell disrupter [192]. Others include homogenizing the sample under liquid nitrogen, combinations of enzymatic treatment, freeze-thaw/boiling and kits for plant DNA purification or for animal tissues DNA purification [192]. For the detection of causative microorganisms in clinical material, specific microbial genomic target sequences are amplified in polymerase chain reaction or other DNA/RNA amplification assays [193].

The polymerase chain reaction (PCR) is an *in vitro* method for the amplification of DNA that was introduced in 1985 [194]. PCR has become established as a useful test for laboratory diagnosis of mycobacteriosis in situations where samples have moderate to large numbers of bacteria and it is important to have a fast diagnosis [195]. With the performance of a previous reverse transcription step, PCR can also be applied to RNA. Reverse transcription PCR is a modification of this method used when the initial template is RNA rather than DNA, the reverse transcriptase enzyme first converts the RNA target into a complementary DNA copy (cDNA), that can be used to amplify the much higher numbers of copies of messenger or ribosomal RNA than the number of DNA copies present in bacteria, and it may detect specific expression of certain genes [196]. The numerous genotyping techniques that have been developed can be classified, based upon the genetic element used, in two categories: the whole genome techniques and the partial genome techniques. The whole genomic techniques, such restriction endonuclease analysis (REA and pulsed field gel electrophoresis (PFGE), were the first to be developed and have the advantage that all the potential genetic information is used. However, the cell wall composition of the mycobacteria renders the organisms relatively

refractive to DNA extraction, with the result that whole genomic based techniques are technically demanding, difficult to automate, and therefore less popular than the techniques that only use part of the genome [197]. In the technique of REA, DNA is cleaved with particular restriction enzymes, the resulting DNA restriction fragments are separated on agarose gels and analysed. Later on, repetitive DNA elements are cloned that could be used as probes to visualize only those restriction fragments that contain the DNA sequence complementary to the probe: RFLP typing [193]. In the technique of PFGE all the steps are the same as for REA, the only difference being the use of restriction endonuclease enzymes to generate a relatively small number of large DNA fragments. Such fragments are too large to be separated by conventional electrophoresis, but can be discriminated when subjected to a constantly changing (pulsed) electrical field. The publications of the complete genome sequences of *M. tuberculosis* H37Rv, *M. bovis* AF2122/97 and *M. bovis* BCG were breakthroughs in molecular TB research. Since then, whole genome sequencing has become more available and has revolutionized genotyping by providing the highest level of discrimination [198]. The partial genome techniques are more commonly used and can be subdivided into (a) techniques that target specific repeated sequences comprising IS, the direct repeat (DR) region, polymorphic GC-rich sequences and tandem repeat *loci*; (b) targeting of random sequences, such as random amplified polymorphic deoxyribonucleic acid (RAPD) analysis; (c) typing targeting housekeeping genes by multilocus sequence typing (MLST); (d) deletion typing targeting RD; and (e) single nucleotide polymorphism (SNP) typing [198]. Standard (housekeeping) genes offer a higher level of sequence variation than do ribosomal genes but are nonetheless useful for taxonomic purposes due to the relative sequence conservation imposed to maintain function. In this category, the heat-shock protein gene 65 (*hsp65*) is a preferred target for mycobacterial identification to the species level, having been routinely used in diagnostics since the development of rapid PCR-restriction enzyme analysis (PRA) methods. The *dnaJ* gene encodes a stress chaperone protein and is highly conserved among the bacterial genera. The sequence of the 16S rDNA gene is specific at the species level and is also a stable property of microorganisms. Targeting the 16S rRNA gene, 3 different probes, specific for mycobacteria, *M. tuberculosis* complex and *M. avium* complex, were constructed and the thermal melting temperature was different for *M. tuberculosis*, *M. kansasii*, *M. avium*, *M. intracellulare* and *M. marinum* allowing the differentiation [199]. Some modifications to single PCR were done to improve results and were used for *Mycobacterium* species detection, the multiplex PCR, the

assay that include several primer pairs specific to different DNA targets to allow amplification and detection of several pathogens at the same time, and nested PCR, in which the product from one PCR reaction serves as template in a second reaction with fresh reagents, thus diluting any PCR inhibiting substances and increasing the sensitivity. As example differentiation of *M. tuberculosis* complex, *M. avium* complex and other NTM has been done by using hybridization probes [200]. One of the major breakthroughs in the study of mycobacterial infections was a discovery of IS in mycobacterial genomes, e.g. IS*6110* and IS*1081* in the MTC strains [161], IS*900* in MAP [201], IS*901* [202], IS*1245* [203], IS*1311* [204] in the MAC strains.

3.4.1 *Mycobacterium tuberculosis* complex

The IS*6110* is considered as specific to the members of the MTC, and the difference in the location and number of copies of this IS is a source of polymorphism between isolates. The classical technique used to observe such polymorphisms is RFLP-IS*6110*, still considered as the reference typing technique for *M. tuberculosis* [205]. This insertion sequence is present in up to 20 copies in *M. tuberculosis*, in contrast, only 1-5 copies of IS*6110* are found in *M. bovis*, which limits the ability of this element to discriminate between different *M. bovis* strains [193]. For the majority of isolates of *M. bovis*, other techniques based on other genetic areas such as the DR region are preferred. The DR region is a monolocus area, virtually specific to the MTC [206]. Each DR region corresponds to the regular repeat of two types of short sequences, sequences which are all identical, called the DR sequences, and sequences which are all different, called the spacers. The association of one spacer and the contiguous DR sequence is called a DVR. The polymorphism between two isolates resides in the fact that one or more spacer(s) can be present/absent in the DR region of one isolate and not of the other. It is also related to the number of DR regions in different isolates. Two different techniques can be applied: RFLP-DR to determine the number of copies of this region, and spoligotyping (for spacer oligotyping) one reverse line blot hybridization technique [207].

Tandem repeat *loci*, similar to eukaryotic minisatellites, have been identified in *M. tuberculosis*. *Loci* with short sequence repeats (SSRs) of 1-3 bp are generally referred to as microsatellites, whilst *loci* with 10-100 bp are referred to as minisatellites. Many of these sequences, which present allelic hypervariability related to the number of repeats and to inter-allelic sequence variability, are called variable number tandem repeats (VNTRs). Indeed, in mycobacteria, the majority of the VNTR correspond to the so-called MIRUs (Mycobacterial Interspersed Repetitive Units) that are minisatellites

structures composed of 51-77 bp sequences, scattered in 41 locations throughout the bacterial chromosome [205]. VNTR-PCR proved to be a robust, convenient, highly discriminatory technique, which is reproducible and appropriate for typing isolates of the MTC, including those with a low IS*6110* copy number [208]. Regions of difference (RD) typing have been used as targets for PCR as a rapid species identification tool. RD typing can be exploited for species differentiation: RD9 is only present in the *M. tuberculosis* and *M. canettii*, which differentiates them for all other MTC members, and RD4 which is absent from all isolates of *M. bovis*. As a result, RDs and additional RDs have been reported: RD1mic, RD1das and RD2seal. Different approaches have been described for the use of three primers, two flanking and one internal [11], or four primers, two flanking and two internal primer pairs [63], to assess the presence or absence of the RDs. Several combinations of PCRs targeting the different RDs have been recommended for species identification: Warren *et al*. [12] have developed a multiplex PCR according to the previously described DNA sequences of the RD. Primer set 1 included RD1, RD4, RD9 and RD12 primers and primer set 2 included RD1mic and RD2seal primers. With this method, using primer set 1, it's possible to differentiate between *M. canettii*, *M. tuberculosis*, *M. caprae*, *M. bovis* and *M. bovis* BCG. The subsequent PCR amplification with primer set 2 enables differentiation of *M. africanum*, *M. microti* and *M. pinnipedii*. Moreover, due to the unidirectional evolution of the RDs [13], these markers are useful for the reconstruction of the evolution of the MTC and to define clonal groups within the MTC host-adapted members [209]. Possible variability in RDs should be considered for deletion typing. Recently, differing results with the RD4 PCR have been described in an Eastern European *M. caprae* isolate, hinting at a possible partial deletion of the *locus* [71]. A multiplex real-time PCR assay targeting RD *loci* has recently been developed [210].

3.4.2 *Mycobacterium avium* complex

When characterized and used in the proper context, the species-specific IS elements can be useful classification tools to distinguish subsets of the MAC [102]. However, there are two problems: first, a number of IS elements have been uncovered in strains considered to be MAC organisms, but without adequate strain characterization, it is difficult to judge which organisms harbor such elements; second, IS elements are by nature mobile elements, so there is a risk that similar elements are found in unrelated bacteria because of mobility to or from MAC organisms [2]. For the identification of *Mycobacterium avium* and *Mycobacterium intracellulare*

specific probes are available. Amplification of DNA targets DT1 and DT6 was considered equally sensitive for species identification. Strains of MAC can be identified by serological procedures, on the basis of differences in the C-mycoside glycopeptidolipids [99]. Shin *et al*. [102] designed a five-target multiplex PCR to discriminate MAC organisms isolated. This MAC multiplex was designed to amplify a 16S rRNA gene target common to all *Mycobacterium* species, a DNA target DT1 that is unique to *M. avium* subsp. *avium* serotypes 2 and 3, to *M. avium* subsp. *silvaticum*, and to *M. intracellulare*, and three insertion sequences, IS*900*, IS*901*, and IS*1311*. The results for the pattern of amplification allowed to determine whether isolates were mycobacteria, or members of MAC, and to classify them into one of the three major MAC subspecies, *M. avium* subsp. *paratuberculosis*, *M. avium subsp. avium*, and *M. avium* subsp. *hominissuis*. MAP genome has revealed the presence of 17 IS*900*, 7 IS*1311*, and 3 IS*1245* insertion elements. The IS*900* element seems unique to MAP and has been widely used as a diagnostic tool to detect MAP in clinical samples from both animals and humans [6]. However, Englund *et al*. [211] recommended that a positive IS*900* PCR should be confirmed by subsequent sequencing or by a PCR assay targeting another gene in MAP. In a study performed by Vansnick *et al*. [212] two sets of newly developed PCR primers based on the insertion sequence IS*900* and the unique sequence f57 were developed and the combination of the two PCR assays has proven to be useful for the identification of MAP. Restriction fragment length polymorphism (RFLP) analysis of the IS*900* element has been used as a molecular tool to type MAP isolates and allowed their division into different groups, associated to different host species [2].

The IS*900* element is by far the most widely used target for the molecular detection of *M. avium* subsp. *paratuberculosis* and has been used in the form of direct PCR [212], *in situ* PCR [213] nested PCR [214], and real-time PCR [215]. Other identification methods of *M. avium* species or its subspecies are PCR-REA [216], sequence analysis of *hsp65* [87] or a strategy based on large sequence polymorphisms [217]. Semret *et al*. [217] evaluating the distribution of genomic polymorphisms across a panel of strains, verified that it was possible to assign unique genomic signatures to host-associated variants and based on these polymorphisms proposed a simple PCR-based strategy that can rapidly type *M. avium* isolates into these subgroups. Morakova *et al*. [218] designed primers specific for the *dnaJ* gene in the *M. avium* species that allow amplification of the *dnaJ* gene in all isolates of all *M. avium* subspecies and the authors suggest using them as an internal standard in the multiplex PCR to control inhibition of the amplification, and

consequently false negatives, because are highly specific for at least *M. avium*. The same team designed a fast and specific PCR strategy for the detection and differentiation of *M. avium* subspecies for use in routine veterinary diagnosis. They have developed a multiplex PCR based on IS*900*, IS*901*, IS*1245* and the *dnaJ* gene. This method allows the detection of *M. a. paratuberculosis*, *M. a. hominissuis* and *M. a. avium* /*M. a. silvaticum* in one PCR reaction and theoretically enables the detection of mixed infections of *M. a. paratuberculosis* and *M. a. avium* or *M. a. paratuberculosis* and *M. a. hominissuis*.

Chapter - 4
Public Health Concerns

The emergence of new infectious diseases in wildlife is the result of interactions between wildlife, domestic animals, and humans. Wild animals are susceptible to infection with many of the same disease agents that affect domestic animals, and transmission between wildlife and domestic animals can occur in both directions [219].

The mycobacteria present in the natural environment and in wildlife are a source of infection to humans, directly or via livestock. The susceptibility of HIV infected individuals to *Mycobacterium* spp. is of major concern to public health officials in developing countries where the acquired immune-deficiency syndrome is rampant [220].

4.1 *Mycobacterium tuberculosis* complex

The bacillus of MTC infects an estimated 2 billion persons worldwide and it is estimated that 1.5 to 2 million people die each year from TB [120]. Ninety-five percent of cases occur in people in developing countries. TB is one of the leading causes of infectious disease-related deaths worldwide [220]. Significant progress has been made toward the elimination of TB caused by MTC from humans in industrialized countries. However, the development of drug-resistant (multidrug-resistant and extensively drug-resistant) strains has compromised the efficacy of TB treatment in humans and has markedly increased the cost associated with the use of multiple drug therapies [221]. It has been noted that certain conditions, such as HIV infection, diabetes, and silicosis predispose to TB. The large use of immune-suppressive drugs, has become a further risk factor for TB. Before starting immune-suppression, an accurate screening for latent TB infection with tuberculin skin testing and immunological tests, such as the interferon gamma release assays (IGRAs), is mandatory [222]. Tuberculosis in mammals, including humans, can be caused by any of the members of the MTC and in the context of One Health, as all members of the MTC can be transmitted between animals and humans (with the potential for spillback) and therefore warrant an interdisciplinary approach [223]. *Mycobacterium bovis* is by far the most important causative agent of TB in livestock and wildlife and is commonly referred to as bovine TB. However, *Mycobacterium tuberculosis*, which is primarily associated

with TB in humans, has also been identified as an emerging threat to domestic and wildlife animal health, especially in countries where human TB is highly prevalent [224]. Although *Mycobacterium bovis* or *M. caprae* infection currently accounts for only a small percentage of reported cases, and zoonotic TB was originally considered primarily as a disease of children where the disease involved the cervical lymph nodes (scrofula), the intestinal tract, or the meninges, it is now increasingly being recognized that infection in childhood is the precursor of reactivated adult disease and that many infected children may remain asymptomatic, undiagnosed, and untreated [225]. *M. bovis* may affect humans of any age, and while the majority opinion is that human to human spread of *M. bovis* must be a very rare event, it does occur particularly amongst immunocompromised individuals [225]. The widespread distribution of *M. bovis* in farm and wild animal populations represents a large reservoir of this microorganism. The spread of the infection from affected to susceptible animals is most likely to occur when domesticated and wild animals share pasture or territory. Well-documented examples of such spread include infection in badgers (*Meles meles*) in the United Kingdom [121], possums (*Trichosurus vulpecula*) in New Zealand [226], red deer in the Alps [227] and wild boar and red deer in Iberian Peninsula [228]. Smith et al. [229] reported, in the United Kingdom, the first documented case of spillover of bovine TB from animals to humans. The *M. bovis* isolated from cattle was indistinguishable from the isolates from the two siblings when examined by RFLP analysis, spoligotyping, and VNTR analysis, suggesting transmission between cattle and humans. Moreover, *M. bovis* infection had been previously diagnosed in a population of badgers on the farm, suggesting that cattle became infected through contact with badgers and that humans became infected through contact with cattle [219]. Tuberculosis in humans due to *M. bovis* is both clinically and pathologically indistinguishable from cases caused by *M. tuberculosis* and the possible reasons for underdiagnosing *M. bovis* in humans are the diagnosis of pulmonary TB usually based on the AFB smear examination; Use of culture is generally reserved for suspected treatment failures and relapses, as well as to measure the prevalence of drug-resistant TB [230]. The potential impact on population groups of *M. bovis* causing human TB at the highest risk should nevertheless not be underestimated. Exposure to aerosol-borne infection with *M. bovis* from cattle and wildlife populations remains highest in farmers, veterinary staff and rural, slaughterhouse workers, and humans through occupational or recreational exposure to wildlife [224]. The introduction of ITT, which marked the beginning of systematic bovine TB control by detecting and eliminating *M. bovis* in combination with the implementation

of compulsory milk pasteurization, probably constituted the biggest and most effective zoonotic TB control [223].

4.2 *Mycobacterium avium* complex

Human exposure to MAC present in wildlife and in nature can occur by a variety of routes. Birds are major excretors of the agent in their faeces and the bacteria can persist in the soil and in water for long. Humans are continuously exposed at a low level (50 to 5000 bacilli per day). Contact with water, municipal or natural are also important routes for mycobacteria infection [96]. Healthy humans have a low susceptibility to MAC infection and only a very small percentage of mycobacteria progress through to infection, but in immunocompromised individuals infected with HIV or leukaemia patients, treated with steroid therapy, chemotherapy or other immunosuppressive medication, MAC causes a variety of disorders including TB-like diseases [96]. Since the advent of AIDS, HIV has become the major risk factor for MAC infection. Prior to the introduction of highly active antiretroviral treatment more than 40% of patients developed *M. avium* complex bacteremia two years after the AIDS diagnosis [231] and a disseminated MAC infection was found in as much as 50% of autopsied AIDS patients [232]. This has predominantly been attributed to the impairment of the adaptive part of the immune systems in HIV-1 infected individuals due to the loss of CD4+ T cells, as the susceptibility to opportunistic infections including *M. avium* infection is correlated with a decline in this cell type. MAC usually produces clinical disease only when CD4+ are very low (< 50 cells/ml), which is seen in 4 to 5 per cent of HIV infected patients. A recent study showed that exposure of dendritic cells to HIV-1 promotes or facilitates the intracellular growth of *M. avium* [232]. Signs and symptoms associated with MAC disease in AIDS cases are persistent high-grade fever, high sweats, anaemia and weight loss in addition to nonspecific symptoms of malaise, anorexia, diarrhea, myalgia and occasional painful adenopathy [172]. In adults, infection is mainly pulmonary [96]. Among the members of MAC, MAA is predominant (87-98%) in AIDS patients [96] and the main route of infection is the gastrointestinal tract and *M. avium* is naturally tolerant to the low pH that exists in the stomach [94]. Epidemiology of MAC complex in patients without HIV infection remains somewhat difficult to determine since the disease is relatively uncommon, however preexisting pulmonary conditions, patients with current illness or immunosuppressive medication are considered the most important risk factors for MAC infection amongst patients without HIV infection. MAC was also reported as the most common pathogen causing post-transplant NTM disease [233]. Other factors are local

traumas and surgical procedures injuries. Chronic obstructive pulmonary disease, emphysema, pneumoconiosis, aspiration due to oesophageal disease, previous gastrectomy and chronic alcoholism are some of the conditions which have been linked to disease [172]. In an epidemiological survey performed in patients without HIV infection, in USA from 2000 to 2003, the rate of positive non-tuberculous cultures was 17.7 per 100,000 and the incidence of NTM in the respiratory tract disease was estimated in 2.0 per 100,000 and the disease in anywhere in the human body in 2.7 per 100,000 [233]. In apparently healthy children, MAC is the most common of the nontuberculous mycobacteria infection and is characterized by a chronic granulomatous lymphadenopathy in the neck region that preferably is treated by excision of the affected lymph node. The main hypothesis of infection is that oral contact with *M. avium*-infected water courses [234]. The zoonotic potential of MAP has been debated for almost a century because of similarities between Crohn´s disease in humans and Johne´s disease in ruminants. More than 25 years later since MAP was first proposed as an etiologic agent in Crohn´s disease, based on the isolation of the organism from several patients, at this moment is not possible to know if MAP is a primary etiological agent or secondary invader and further research is needed to understand the possible links between this agent and Crohn's disease [235].

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