

Chapter 1

Literature review

1.1 History

Phthisis is what the Greeks (ca 460 BC) called tuberculosis (TB) and it means to waste away. It was very appropriately named since emaciation is one of the most prominent clinical signs of human and bovine TB (BTB) (Herzog 1998). It was not until 1865 that Villemin (1827-1892), a French military doctor, discovered that the disease in humans or cattle could be transmitted to rabbits (Herzog 1998; Daniel 2006). By performing an experiment in animals he injected blood or sputum from humans and cattle into laboratory rabbits and found that they developed TB, whereas a similar transfer of cancerous tissue displayed no effect on the recipients (Sakula 1983). At this stage the causative agent was not yet identified. Discovering that the disease was communicable led to the understanding that the disease is caused by a specific micro-organism. These findings by Villemin were published in *Comptes rendus de l'Academie des Sciences* (Sakula 1983). Robert Koch (1882) discovered the tubercle bacillus but did not differentiate between the isolates from cattle and humans (Koch 1882; Grange & Bishop 1982). It was Professor Theobald Smith who, in 1898, differentiated between the two tubercle bacilli and noted that the designation of the two types did not imply that they were restricted to the hosts after which they were named (Sakula 1983).

During the early 1900s there was much controversy about the importance of the bovine tubercle bacillus as a source of human disease. Koch proclaimed that humans were immune or only slightly susceptible and that control measures in cattle were unnecessary (Sakula 1983). Koch, however, failed to convince many people from the veterinary profession and M'Fadyean, Ravanel and Bang (Grange & Bishop 1982) along with other researchers started obtaining data regarding this issue. Ten years later results of various researchers refuted Koch's views of *Mycobacterium bovis* and established the risk of bovine tubercle bacilli to human health, and confirmed that milk is the principal source of transmission. Measures were put in place and were made effective by the policy of eradication of infected cattle and the pasteurization of milk (Sakula 1983). The discoveries of Robert Koch and other famous scientists who contributed significantly to mycobacteriology have stimulated others to search

for preventative and curative modalities, and the development of diagnostic techniques for both TB and BTB.

1.2 Mycobacteria of the *Mycobacterium tuberculosis* complex (MTBC)

Mycobacteria belong to the order Actinomycetales, family Mycobacteriaceae, and genus *Mycobacterium* (Table 1.1). Mycobacteria of the MTBC are aerobic, non-motile bacilli with a thick prominent cell wall which is rich in mycolic acids and is hydrophobic (Rastogi, Legrand & Sola 2001). Due to this structural characteristic the Gram staining technique cannot be used to assist identification. Instead they are referred to as acid-fast bacteria because once they are stained with the Ziehl-Neelsen staining method (Van Deun, Hossain, Gumusboga & Rieder 2008) they resist decolouration with acid-alcohol. These mycobacteria are slow growers with a doubling time of 18 to 24 hours. They grow optimally at 37°C (Karlson & Lessel 1970; Rastogi *et al.* 2001).

Table 1.1 Classification of the *Mycobacterium tuberculosis* Complex (Bergey & Holt 1993)

Kingdom	Bacteria
Phylum	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	<i>Mycobacterium</i>
Species	<i>M. tuberculosis</i> , <i>M. bovis</i> , <i>M. caprae</i> , <i>M. microti</i> , <i>M. pinnipedii</i> , <i>M. africanum</i>

The MTBC encompasses the following species: *M. tuberculosis* (Rastogi *et al.* 2001) the human tubercle bacillus; *M. bovis* (Karlson & Lessel 1970) the bovine tubercle bacillus; *M. microti* (Reed 1957) a rare pathogen of rodents and other small mammals; *M. africanum* causing TB in humans mainly in equatorial Africa; *M. pinnipedii* (Cousins, Francis, Gow *et al.* 1990; Cousins, Bastida, Cataldi *et al.* 2003) primarily infecting seals. *Mycobacterium caprae* (Aranaz, Cousins, Mateos & Dominguez 2003), initially classified as a subspecies of *M. bovis*, was recently recognised as a species on its own. This pathogen primarily infects goats but cattle, pigs and humans are also susceptible (Cvetnic, Katalinic-Jankovic, Sostaric *et al.* 2007).

Modern genome analysis of the MTBC has revealed, contrary to previously held dogma, that human TB has not evolved from *M. bovis* (Brosch, Gordon, Marmiesse *et al.* 2002). A separate lineage that is represented by *M. africanum*, *M. microti* and *M. bovis* branched from the progenitor of *M. tuberculosis* strains. This process was accompanied by a successive loss of DNA, which may have contributed to the appearance of more successful pathogens in new host species. *Mycobacterium canettii*, a rare tubercle bacillus could represent the most ancestral lineage of the MTBC (Brosch *et al.* 2002). *Mycobacterium canettii* and other smooth tubercle bacilli actually correspond to lineages belonging to a much broader progenitor species from which the MTBC emerged. This last common ancestor could already have affected early hominids in East Africa at least 2.6 million years ago (Gutierrez, Brisse, Brosch *et al.* 2005).

1.3 Tuberculosis in humans

Tuberculosis, caused by *M. tuberculosis*, is a major disease in humans and is now the leading cause of death in adults worldwide as a result of the human immunodeficiency virus (HIV) epidemic. The World Health Organisation (WHO) estimates, 2 billion people, about one-third of the world's population, are infected with *M. tuberculosis* (WHO 2008). In 2003 about 8.8 million people were estimated to have developed TB (incidence rate of 140 per 100 000 population), of whom 1.7 million were fatalities (mortality rate 28 per 100 000 population) with 99% of these being concentrated in developing countries, particularly Asia and Africa (Figueroa-Munoz, Palmer, Poz *et al.* 2005; Gunneberg, Reid, Williams *et al.* 2008). This situation is believed to be closely associated with the spread of HIV in developing countries. It is estimated that 70% of the 24 million people that are globally co-infected with HIV and *M. tuberculosis* live in sub-Saharan Africa (Corbett, Watt, Walker *et al.* 2003).

In 2002 it was estimated that in India 4.58 million people were co-infected (HIV-TB) (Narain & Lo 2004) and at present approximately 5% of new *M. tuberculosis* infections occur in people with HIV infection (Steinbrook 2007). Furthermore, the spread of TB is intensified by poor sanitary and living conditions due to poverty and the delay in acting against the infection (WHO 2008). Tuberculosis is a contagious disease. Individuals with TB lung lesions spread the infection by aerosol when coughing, sneezing, talking or spitting (Harries & Dye 2006a). Inhalation of a small number of tubercle bacilli is sufficient to induce infection in the individual. During the initial infection when the mycobacteria enter the body,

they settle on the host tissue, taking several weeks to multiply. The immune system recognises the foreign invasion and a cell mediated immune response is elicited. The cells of the tissues or organs, which are infected, multiply to “wall off” the bacteria thus preventing further spread of the pathogen. At this stage of infection a small tuberculous nodule is formed, called a tubercle. The tubercle is encapsulated by connective tissue and the bacteria become dormant. When the individual's immune system is weakened the pathogen multiplies and spreads to infect other regions of the lungs and body. This can lead to active pulmonary TB and, if not treated, can lead to the individual's death (Glickman & Jacobs 2001). Each person with active TB will infect on average between 10 and 15 people every year. Not all people infected with TB bacilli will necessarily develop clinical symptoms of the disease (van Lerberghe, Evans, Rasanathan & Mechbal 2008). People co-infected with HIV and TB bacilli infections are much more likely to develop frank TB. In most cases, if individuals with latent TB become infected with HIV, reactivation and progression to active TB disease occurs (Gunneberg *et al.* 2008; Pai & O'Brien 2008).

Humans are also susceptible to infection with *M. bovis*. This infection may lead to zoonotic TB (Fritsche, Engel, Buhl & Zellweger 2004). Humans may be exposed to the pathogen by consumption of unpasteurized cow's milk and its products or through close physical contact with infected cattle. Transmission of *M. bovis* between humans is rare and anecdotal, and it is clearly less relevant as compared to animal-to-human transmission (Wedlock, Skinner, de Lisle & Buddle 2002). The transmission of TB between humans due to *M. bovis* is better documented in industrialized than in developing countries (de la Rua-Domenech 2006a).

Bovine TB is more common in rural dwellers as compared to urban dwellers (Ayele, Neill, Zinsstag *et al.* 2004), that inhale dust particles containing bacteria or aerosols shed by infected animals which may give rise to pulmonary TB. Urban dwellers are more likely to suffer from extra-pulmonary TB because they acquire the infection via the gastrointestinal route (Ayele *et al.* 2004).

The prevalence of zoonotic TB caused by *M. bovis* in African and Asian countries is much higher than elsewhere in the world, and the problem is further complicated by the alarming spread of HIV in these countries (Narain & Lo 2004). Complementing this setback are the constraints in the control and diagnosis of TB (Ayele *et al.* 2004; Regassa, Medhin & Ameni 2008) as control measures and diagnostic techniques are either inadequate or, in most cases, unavailable (McCrindle & Michel 2006). *Mycobacterium bovis* was confirmed in seven of 65

(10.8%) human cervical adenitis cases in Tanzania and Uganda (Cleaveland, Shaw, Mfinanga *et al.* 2007; Oloya, Opuda-Asibo, Kazwala *et al.* 2008). In Nigeria, a recent study indicated that 5% of human isolates were found to be *M. bovis* (Cadmus, Palmer, Okker *et al.* 2006). In the UK, where the incidence of BTB is likely to be under-reported (de la Rua-Domenech 2006a), *M. bovis* represents approximately 1.1 to 1.5% of bacteriologically proven TB cases (Rowe & Donaghy 2008). The fact that *M. bovis* does contribute to the current human epidemic of TB emphasizes the importance of increasing awareness of zoonotic TB in order to implement appropriate veterinary public health control measures.

1.3.1 Diagnosis of tuberculosis in humans

Diagnostic approaches are categorized into pathogen identification and assessment of the host's immunological response to infection. Conventional TB diagnosis relies on tests such as sputum smear microscopy, culture, intradermal tuberculin tests (IDT) and chest radiography (Harries & Dye 2006a; Harries, Boxshall, Phiri & Kwanjana 2006b; Pai & O'Brien 2008). Culture and microscopy techniques are routinely used to identify the pathogen but are not necessarily advantageous to making an individual diagnosis. This is because the *M. tuberculosis* organism takes 2-3 months to become visible on culture media and such cultures are usually also used for monitoring drug sensitivity patterns in patients with recurring TB (Harries & Dye 2006a; Harries *et al.* 2006b). Another drawback is the low sensitivity of the techniques since infected individuals not shedding the pathogen will not be detected. Apart from this limitation the above-mentioned tests perform poorly in populations infected with the HIV (Pai & O'Brien 2008). The vast majority of TB patients live in low and middle income countries (Dye, Watt, Bleed *et al.* 2005). The diagnosis of TB disease, in such countries, relies primarily on the identification of acid-fast bacilli in unprocessed sputum smears using a conventional light microscope and chest X-rays, which are used to diagnose pulmonary TB.

Microscopy is used mostly for *M. tuberculosis* detection in TB-endemic countries (Toman 2004; Steingart, Henry, Laal *et al.* 2007). Microscopy has been reported to have variable sensitivity: low in some reports and high in others (range 20% to 80%) (Urbanczik 1985; Steingart, Henry, Hopewell *et al.* 2006). More importantly, sensitivity is poor for paucibacillary disease (e.g. pediatric and HIV-associated TB) (Shingadia & Novelli 2003). This lack of sensitivity of the sole diagnostic test in many parts of the world results in delays

in diagnosis, enabling the disease to progress and increasing the potential for transmission of *M. tuberculosis* (Behr, Warren, Salamon *et al.* 1999). To ensure appropriate measures to improve the control of the global TB epidemic, simple, accurate, inexpensive and, ideally, point-of-care diagnostic tools for TB are urgently needed. The choice of a diagnostic test depends upon the setting in which the test is to be performed and the intended use of the results (fit for purpose principle). If test results are to be used to exclude TB in patients with respiratory symptoms in TB-endemic countries, then tests with a high sensitivity (and thus providing high negative predictive value) are required even if the test is only moderately specific. Once a smaller “at risk” group is identified, a rigorous diagnostic work-up will be performed. In this case patients with respiratory symptoms for anti-TB treatment should be identified; therefore a test with a high specificity (and thus providing high positive predictive value) is required. A recent systematic review on commercial serological antibody detection tests for the diagnosis of pulmonary TB highlighted that none of the commercial tests evaluated performed well enough to replace sputum smear microscopy (Steingart *et al.* 2007). Thus nowadays, these commercial serological tests have little or no role in the diagnosis of pulmonary TB.

The IDT relies on the response of the individual to the injection of tuberculin and is described as a delayed type hypersensitivity (DTH) response. The test has several drawbacks, some of which include the return visit of the patient to allow reading of the test, problems in interpreting the results, variability in its application and readings, immunosuppression, and cross-reactivity in Bacille Calmette-Guérin (BCG) vaccinated people (Pottumarthy, Morris, Harrison & Wells 1999). Furthermore the IDT is not 100% specific or sensitive, and an average of 20-25% of patients with active TB do not react to the purified protein derivative (PPD) used in the IDT (Fietta, Meloni, Cascina *et al.* 2003). Recently *in vitro* tests measuring cell mediated immunity (CMI) have been developed. These include interferon-gamma release assays (IGRAs), such as the Quanti-FERON[®]-TB (QFT) Gold (Cellestis Inc., Victoria, Australia) and T_SPOT.TB (T_SPOT, OXFORD Immunotec). The QFT Gold and the immunospot-formatted T_SPOT measure the IFN- γ produced by T-cells in whole blood upon stimulation by *M. tuberculosis* antigens. Diel, Loddenkemper, Meywald-Walter *et al.* (2008) compared the performance of IDT, QFT test and T_SPOT test in contact investigations for TB. The authors concluded that IGRAs are more accurate indicators of the presence of latent TB infections (LTBI) than IDT. They also noted that IGRAs show excellent agreement with

each other. Other researchers (Ozdemir, Annakkaya, Tarhan *et al.* 2007; Manuel & Kumar 2008; Nienhaus, Schablon & Diel 2008) also showed that the QFT test is an ideal test for the diagnosis of LTBI. Quanti-FERON[®]-TB Gold shows a higher sensitivity for detecting active TB (i.e. sputum positive patients) than the IDT (Kobashi, Obase, Fukuda *et al.* 2006) and is considered as a useful diagnostic aid for the diagnosis of active TB in humans when compared to IDT (Bartu, Havelkova & Kopecka 2008). A recent report suggests that active TB can be clearly identified provided that T-cell IGRAs are performed on mononuclear cells derived from the site of disease (Jafari & Lange 2008).

1.4 Tuberculosis in animals

Mycobacterium bovis is the principal cause of BTB and the success of this pathogen is reflected by its global distribution in a diversity of mammalian host species, including domestic, captive and free-ranging animals, and humans. The impact of this disease is not restricted to the health of the species but also has economic implications because of the production and trade of these animals, and for ecotourism. Reservoir wildlife hosts have become a source of infection for domestic animals (Corner 2006) and thus may affect entire ecosystems (Renwick, White & Bengis 2007). Maintenance hosts of *M. bovis* include the African buffalo (*Syncerus caffer*) in South Africa (Bengis, Kriek, Keet *et al.* 1996); the European badger (*Meles meles*) in the UK (Nolan & Wilesmith 1994); the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Corner, Stevenson, Collins & Morris 2003); bison (*Bison bison*) in Canada (Lutze-Wallace, Turcotte, Stevenson *et al.* 2006) and white-tailed deer (*Odocoileus virginianus*) in the USA (Schmitt, Fitzgerald, Cooley *et al.* 1997).

Animal-to-animal transmission of *M. bovis* can occur via contact, aerosol exposure and ingestion of contaminated material. Contact between animals can occur at water points and places where animals are likely to gather (milking parlours, transportation, market places, dipping tanks, auction stations and grazing areas) (Renwick *et al.* 2007). Aerosol exposure to *M. bovis* is considered to be the most frequent route of infection of cattle and results in the entry of the pathogen into the host.

Tuberculosis, caused by *M. tuberculosis* is not restricted to humans, its main host. According to present knowledge, *M. tuberculosis* does not appear to have an animal reservoir and the animals that become infected most probably represent accidental hosts (Thoen, Steele &

Gilsdorf 2006). For transmission of *M. tuberculosis* infection to susceptible animals there has to be prolonged and close contact with humans (Michel, Bengis, Keet *et al.* 2006; Parsons, Gous, Warren & van Helden 2008). Disease associated with *M. tuberculosis* has occurred mostly within captive settings. In countries, such as India (Srivastava, Chauhan, Gupta *et al.* 2008), Ghana (Bonsu, Laing & Akanmori 2000), Nigeria (Mawak, Gomwalk, Bello & Kandakai-Olukemi 2006), Slovenia (Pavlik, Ayele & Parmova 2003; Ocepek, Pate, Zolnir-Dovc & Poljak 2005) and the UK (de la Rua-Domenech 2006a), infection with *M. tuberculosis* has been most frequently identified in cattle. Published data also indicates that the prevalence of *M. tuberculosis* in cattle herds did not exceed 1% in the majority of studies (Ocepek *et al.* 2005). A prevalence of 6.2% and 7.4% was, however, recorded in Algeria (Boulahbal, Benelmouffok & Brahimi 1978) and Sudan (Suliman & Hamid 2002) respectively. Other cases of *M. tuberculosis* in domestic animals includes cats (*Felis catus*) (Gunn-Moore 1994; Fernandez & Morici 1999), domestic pigs (*Sus scrofa f. domestica*) (Pavlik *et al.* 2003), and canines (*Canis lupus*) (Aranaz, Liébana, Pickering *et al.* 1996; Turinelli, Ledieu, Guilbaud *et al.* 2004; Parsons *et al.* 2008). *Mycobacterium tuberculosis* has been reported in both Asian (*Elephas maximus*) (Mikota, Larsen & Montali 2000) and African (*Loxodonta africanum*) (Gorovitz 1962) elephants. In free-living mammals it was thought to be absent (Montali, Mikota & Cheng 2001) until it was reported in free-ranging banded mongooses (*Mungos mungo*) in Botswana and suricates (*Suricata suricatta*) in South Africa (Alexander, Pleydell, Williams *et al.* 2002), its occurrence being believed to be associated with ecotourism.

1.4.1 Cattle

In countries which have instituted comprehensive eradication programmes, the clinical disease in cattle is rarely seen because the IDT enables a presumptive diagnosis to be made and elimination of infected animals to be effected before the disease is advanced and clinical signs are recorded. Prior to the national TB eradication campaigns, however, the clinical signs associated with TB were commonly observed (de Lisle, Mackintosh & Bengis 2001).

1.4.2 Free-ranging wildlife

In South Africa, *M. bovis* infections were first reported in 1929 in greater kudu (*Tragelaphus strepsiceros*) and in a common duiker (*Sylvicapra grimmii*) (Cousins 2008). In Uganda, a 10% prevalence of BTB in African buffaloes and 9% prevalence in warthogs (*Phacochoerus aethiopicus*) were reported in 1982 (Woodford 1982). *Mycobacterium bovis* infections in wildlife in Zambia, Kenya, Tanzania (Cleaveland, Mlengeya, Kazwala *et al.* 2005; Cousins 2008) and later in the Kruger National Park (KNP) of South Africa were reported. In South Africa, the disease has already spilled over into chacma baboons (*Papio ursinus*), lions (*Panthera leo*), cheetahs (*Acinonyx jubatus*), leopards (*Panthera pardus*), honey badgers (*Mellivora capensis*), hyenas (*Crocuta crocuta*) (Keet, Kriek, Penrith *et al.* 1996; Keet, Kriek, Bengis *et al.* 2000; De Vos, Bengis, Kriek *et al.* 2001), warthogs (*Phacochoerus aethiopicus*), kudu (*Tragelaphus strepsiceros*) (Keet, Kriek, Bengis & Michel 2001), and a bush pig (*Potamochoerus larvatus*) (Michel, Coetzee, Keet *et al.* 2009).

In North America, *M. bovis* has been detected in white-tailed deer (*Odocoileus virginianus*) in USA, in the northeastern part of Michigan state (O'Brien, Schmitt, Fierke *et al.* 2002), in Canada in elk (*Cervus elaphus*) in Manitoba province (Fuller 1959; Nishi, Shury & Elkin 2006), and wood bison (*Bison bison athabascae*) in Alberta province (Lutze-Wallace *et al.* 2006). *Mycobacterium bovis* has spread to wildlife populations in many European countries. In Spain, it has been reported in wild boars (*Sus scrofa*), Iberian lynx (*Lynx pardinus*) and deer species (Gortazar, Torres, Vicente *et al.* 2008). In Great Britain and Ireland, the extent of *M. bovis* infections in the badger population and its role in the increase of TB caused by *M. bovis* in cattle is still a matter of vigorous debates (Courtenay, Reilly, Sweeney *et al.* 2006; White, Bohm, Marion & Hutching 2008). Australia has been successful in eradicating *M. bovis* from cattle herds but there are populations of animals such as feral pigs and feral Asian water buffaloes (*Bubalus bubalis*) that have been reported to be infected with *M. bovis* in the northern parts of the country (Corner, Barrett, Lepper *et al.* 1981; McInerney, Small & Caley 1995). As in South Africa, New Zealand has a diversity of wild animals infected with *M. bovis*. Apart from the reservoir host, which is the brushtail possum, *M. bovis* infections have been reported in feral populations of cats, ferrets (*Mustela putorius*) and stoats (*Mustela erminca*) in Otago and Southland provinces (Ragg, Moller & Waldrup 1995). The continuous transmission of *M. bovis* from free-ranging wildlife reservoirs to domestic livestock results in

economic losses and is a significant barrier to the success of national eradication and control programmes worldwide.

1.4.3 Captive wildlife with special emphasis on rhinoceroses and elephants

Disease associated with *M. tuberculosis* infection has been diagnosed most frequently in animals in captive situations. It has been reported in snow leopards (*Uncia uncia*) (Helman, Russell, Jenny *et al.* 1998), Asian (Furley 1997; Mikota *et al.* 2000; Payeur, Jarnagin, Marquardt & Whipple 2002) and African elephants, terrestrial tapirs (*Tapirus terrestris*), agoutis (*Dasyprocta aguti*) (Pavlik *et al.* 2003), rhinoceroses (Valandikar & Raju 1996; Oh, Granich, Scott *et al.* 2002) and ungulates such as oryx (*Oryx gazelle beisa*), black buck (*Antelope cervicapra*), bongo antelope (*Tragelaphus eurycerus*), mountain goats (*Oreamnos americanus*) and giraffes (*Giraffa camelopardlis*) (Montali *et al.* 2001). Cases of *M. tuberculosis* infections have been diagnosed in Asian elephants in zoo collections in the USA and Europe, and in circuses in the USA (Mikota *et al.* 2000; Pavlik *et al.* 2003; Lewerin, Olsson, Eld *et al.* 2005).

Animals in captivity or in domesticated situations are also susceptible to infection by *M. bovis*. *Mycobacterium bovis* infections have been reported in rhinoceroses (Mann, Bush, Janssen *et al.* 1981), baboons (*Papio hamadryas*), leopards (*Panthera uncia* and *Panthera pardus*) (Thorel, Karoui, Varnerot *et al.* 1998), European wild boars (*Sus scrofa*) and Iberian red deer (*Cervus elephas hispanicus*) (Gortazar *et al.* 2008). There have been no reports of *M. bovis* infection in captive or free-ranging African elephants. Tuberculous captive wildlife maintained on farms or animal parks (e.g. zoos or game farms) may also serve as foci of infection for domestic animals, free-ranging wildlife, and humans (Bengis *et al.* 1996; de Lisle *et al.* 2001; Moller, Röken, Petersson *et al.* 2005).

1.4.3.1 Rhinoceroses

Tuberculosis caused by either *M. bovis* (BTB) or *M. tuberculosis* (TB) has not been reported in free-living rhinoceroses. All cases of the disease in rhinoceroses have been reported in captive situations. In 1981, *M. bovis* infections were reported in captive black rhinoceroses (*Diceros bicornis*) in a zoo in the USA (Mann, Bush, Janssen *et al.* 1981). At necropsy the major lesions (nodular-like) were found in the lungs. On a histopathological level the lungs

contained multiple granulomas with giant cells and areas of alveolar wall fibrosis (Mann *et al.* 1981). In another zoo situation in the USA, Dalovisio, Stetter & Mikota-Wells (1992) reported the exposure of seven zookeepers to a southern white rhinoceros (*Ceratotherium simum simum*) infected with *M. bovis*. This animal was presumably infected via aerosols generated during cleaning of the barn in which the rhinoceros was kept. All the zookeepers who were exposed to infection demonstrated conversion by the skin test, but, apart from rhinorrhoea, none had clinical illness. In South Africa, the first case of mycobacteriosis in a black rhinoceros was diagnosed in 1970 in the Hluhluwe-iMfolozi Park (HiP) (Keep & Basson 1973).

During 1989 and 1991 at a zoo in Louisiana, seven animal handlers who were previously negative for TB tested (Mantoux) positive after an *M. bovis* outbreak in a southern white rhinoceros and monkeys (Stetter, Mikota, Gutter *et al.* 1995). Two black rhinoceroses died in 1992 and 1994 respectively at Mysore zoo in India from pulmonary TB. Post-mortem findings from the first rhinoceros were enlarged, oedematous lungs. The intralobular septa were thickened, and multiple nodules and abscesses were found on the entire surface of the lungs. In the second rhinoceros the lungs had granulomas and the liver was shrunken and necrosed with multiple nodules. The cause of the death, which was confirmed by bacteriological examination, indicated that *M. tuberculosis* was the infectious organism (Valandikar & Raju 1996). In September 1998 *M. tuberculosis* was cultured from a captive black rhinoceros but humans were not found to be responsible for the infection as genotyping evidence suggested that transmission was from another animal species in the zoo (Oh *et al.* 2002).

1.4.3.2 Elephants

Tuberculosis in Asian elephants has been reported since the 1930s (Baldrey 1930). Between August 1996 and May 2000 all isolates of elephant TB in North America were identified as *M. tuberculosis* (Mikota *et al.* 2000; Montali *et al.* 2001). Tuberculosis in captive and domestic Asian elephants has been reported in the USA, Europe and Asia (Pavlik *et al.* 2003; Lewerin *et al.* 2005), (Table 1.2). In 1996, 12 circus Asian elephant handlers were infected with *M. tuberculosis* on an exotic animal farm in Illinois, one of them showing signs consistent with the active disease after three elephants had died of TB. Medical history and testing of the handlers indicated that the elephants had been the probable source of exposure

for most of the human infections (Michalak, Austin, Diesel *et al.* 1998). In addition an outbreak of *M. tuberculosis* in Asian elephants occurred in a Swedish zoo between 2001 and 2003 (Lewerin *et al.* 2005). Five elephants were found to have been infected by four different strains of *M. tuberculosis*. In 1997, the United States Department of Agriculture (USDA) developed guidelines (<http://www.elephantcare.org/protodoc>) for the taking of samples from elephants for bacterial culture by using a trunk-wash method, and for the removal of TB-infected elephants from public contact (Mikota, Miller, Dumonceaux *et al.* 2008). These guidelines were revised in 2003 and again in 2008.

Clinical signs in captive elephants infected with *M. tuberculosis* include loss of appetite and weight, reluctance to do strenuous work and, in some, subcutaneous ventral oedema (Mikota *et al.* 2000; de Lisle, Bengis, Schmitt & O'Brien 2002). In some cases infected elephants show no clinical signs of disease (Lysahchenko, Greenwald, Esfandiari *et al.* 2006). Pathological examinations have indicated that the lungs, bronchi, trachea and the thoracic lymph nodes are primarily involved when the infection involves *M. tuberculosis* (Mikota *et al.* 2000; Montali *et al.* 2001; Lewerin *et al.* 2005). Characteristic histological findings include epithelioid granulomas with significant giant cell formation in the earlier stages. Lymph node and pulmonary lesions exhibiting extensive caseous and pyogranulomatous reactions occur in the advanced form of the disease. The presence of mycobacteria in the areas of caseation in the lungs is common, but is typically rare in the lymph nodes (Montali *et al.* 2001). Tuberculous elephants with extensive involvement of both lungs usually die and show severe caseo-calcareous and cavitating lesions as well as abscesses in the lungs on post-mortem examination. In the extensive cases, firm granulomatous nodules occur in the bronchial lymph nodes which are extensively enlarged and usually show a proliferative response with less caseation than the pulmonary lesions (Mikota *et al.* 2000; Lewerin *et al.* 2005).

Table 1.2 Reported incidence of TB in African and Asian elephants

Cases of Tuberculosis	Reference
<i>M. tuberculosis</i> in an Asian elephant	(Narayana 1925)
<i>M. tuberculosis</i> in an Asian elephant	(Bopayya 1928)
<i>M. tuberculosis</i> in an Asian elephant	(Baldrey 1930)
<i>M. tuberculosis</i> in an African elephant in a zoo in Paris	(Urbain & Dechambre 1937)
<i>M. tuberculosis</i> in an African elephant in a zoo	(Gorovitz 1962)
Fatal tuberculosis pneumonia in an elephant	(Seneviratna, Wettimuny & Seneviratna 1966)
<i>M. tuberculosis</i> in a domestic Asian elephant	(Pinto, Jainudeen, Panabokke <i>et al.</i> 1973)
<i>M. tuberculosis</i> in an Asian elephant	(Johnston 1981; Gutter 1981)
Pulmonary <i>M. tuberculosis</i> in an Asian elephant (circus elephant)	(Saunders 1983)
<i>M. tuberculosis</i> in Asian elephants (captive and circus elephants)	(Binkley 1997; Furley 1997; Whipple, Meyer, Berry <i>et al.</i> 1997)
<i>M. tuberculosis</i> in Asian elephants reported during 1994-1996 at an exotic animal farm Illinois, USA	(Michalak <i>et al.</i> 1998)
<i>M. tuberculosis</i> in 18 Asian elephants during August 1996-May 2000 in North America	(Mikota <i>et al.</i> 2000)
<i>M. tuberculosis</i> culture-positive elephants	(Harr, Raskin, Blue & Harvey 2001)
Mycobacterial isolations from captive Asian elephants and <i>M. tuberculosis</i> infection in an Asian elephant	(Payeur <i>et al.</i> 2002)
<i>M. tuberculosis</i> in an African elephant in Gdansk Zoo, Poland	(Pavlik <i>et al.</i> 2003)
<i>M. tuberculosis</i> in 2 Asian elephants in Assam, India	(Sarma, Bhawal, Yadav <i>et al.</i> 2006)
Outbreak of <i>M. tuberculosis</i> infection in captive Asian elephants in a Swedish Zoo	(Lewerin <i>et al.</i> 2005)
<i>M. szulgai</i> (atypical mycobacterium) in an African elephant at Lincoln Park Zoo, USA	(Lacasse, Terio, Kinsel <i>et al.</i> 2007)

1.5 Diagnosis of *M. bovis* and *M. tuberculosis* infections in animals

1.5.1 Post-mortem

Post-mortem diagnosis of *M. bovis* infection in cattle and bacteriological examination of samples are important steps in the diagnosis of BTB. Lesions that are found during necropsy are collected and a presumptive diagnosis using histopathology is performed. However, for a definitive diagnosis, the isolation of *M. bovis* is required (Corner 1994). For post-mortem diagnosis the method that is employed and the anatomical sites that are examined are considered important as they can affect the effectiveness of the examination (Corner, Melville, McCubbin *et al.* 1990; Corner 1994). In cattle, which have reacted positively to the IDT, essential tissues such as the mediastinal, medial retropharyngeal and bronchial lymph nodes, palatine and pharyngeal tonsils and lungs as well as the parotid, caudal cervical and superficial inguinal and mesenteric lymph nodes should be examined in order to identify all the tuberculous cattle (Corner *et al.* 1990; Corner 1994; Cassidy, Bryson & Neill 1999). In wildlife species, such as deer, tuberculous lesions are not pathognomonic of *M. bovis* infection due to gross and microscopic similarities with lesions caused by *Mycobacterium avium subsp. paratuberculosis* or *M. avium subsp. avium* (Godfroid, Delcorps, Irengé *et al.* 2005). In elephants and rhinoceroses, major lesions are found in the lungs. Hence the “gold standard” for the diagnosis of TB / BTB remains the isolation by culture and identification of *M. bovis* or *M. tuberculosis*.

1.5.2 Identification of the bacteria and / or their genetic material

A presumptive diagnosis of *M. bovis* / *M. tuberculosis* infections can be made on microscopic detection of acid-fast bacilli in smears of suspect tuberculous lesions stained by the Ziehl-Neelsen method (Van Deun *et al.* 2008). However, the absence of acid-fast organisms in a smear does not necessarily rule out a diagnosis of TB. The definitive diagnosis requires that the *M. bovis* organism be isolated from the infected tissue. A disadvantage of the latter is that *M. bovis* grows slowly in culture, taking from 4 to 6 weeks to form visible colonies. A further disadvantage of using this means of diagnosis is the resulting delay in action in managing the problem or infection by causing delays in locating potentially exposed animals. In addition, isolates have to be confirmed by biochemical evaluation, polymerase chain reaction (PCR) and other molecular typing techniques. The PCR technique, although not superior to routine culture in terms of sensitivity, specificity or reliability, does allow the rapid screening of

samples from live animals, monitoring of the environment for the presence of mycobacteria (de la Rua-Domenech, Goodchild, Vordermeier *et al.* 2006b) and classification of mycobacteria into the MTBC group or the *Mycobacterium avium* complex (Miller, Jenny & Payeur 2002). An added advantage of PCR over culturing techniques is that it does not require the presence of viable organisms. Insertion sequences, IS6110 and IS1081, are specific to the mycobacteria belonging to the MTBC, and these insertion sequences are used in PCR techniques to determine the presence of *M. tuberculosis* and *M. bovis* in cattle with suspected TB (Skuce, McCorry, McCarroll *et al.* 2002). Recently, a two-step, multiplex PCR method based on genomic regions of difference was developed for the differentiation of members of the MTBC. The size of the respective PCR amplification products correspond to the presence of the different *M. tuberculosis* complex members (Warren, Gey van Pittius, Barnard *et al.* 2006).

1.5.3 Genotyping of mycobacteria

Before the development of molecular typing techniques, the available techniques of strain typing did not allow the differentiation of *M. bovis* isolates (Haddad, Monique & Durand 2004). Molecular techniques have proven to be very useful as tools for differentiating not only strains that belong to the MTBC but also between strains within the species (Gutierrez, Samper, Gavigan *et al.* 1995), thus making it possible to determine the origin of outbreaks, understand the link between different outbreaks, show relationships between domestic and wildlife TB, and also identify sources of infection (Haddad *et al.* 2004). Typing techniques used for *M. bovis* strain typing include restriction enzyme analysis (REA) (Collins & de Lisle 1985), polymorphic (GC)-rich sequences (PGRS) (Ross, Raios, Jackson & Dywer 1992), restriction fragment length polymorphism (RFLP) analysis (Skuce, Brittain, Hughes & Neill 1994), spoligotyping (Gutierrez *et al.* 1995; Kamerbeek, Schouls, Kolk *et al.* 1997; Roring, Brittain, Bunschoten *et al.* 1998) and variable number of tandem repeats (VNTRs) (Le Fleche, Fabre, Denoeud *et al.* 2002)

1.5.3.1 Restriction enzyme analysis

In this technique high molecular weight DNA is digested with a restriction enzyme and the fragments produced are separated by gel electrophoresis. Strains are characterized on the

basis of the fragment patterns (Collins & de Lisle 1985). This technique differentiates between strains and isolates.

1.5.3.2 Polymorphic (GC)-rich sequences

Polymorphic (GC)-rich sequences are sequences with an 80% GC content and belong to the “PE multigene family”. Multiple copies of these highly homogenous genes are present in mycobacteria. For typing, polymorphism is based on the number and location of the PGRS domains and is determined using PGRS-RFLP analysis (Haddad *et al.* 2004; Michel *et al.* 2009). Upon analyses, a distinction can be made between different isolates of the same species.

1.5.3.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism takes place in two steps. In the first, a part of a gene common to all species of the MTBC is amplified using specific primers. These primers do not generate PCR products from other species of mycobacteria. This is followed by the second step in which the amplicons produced are digested with restriction enzymes. Upon analyses of the products, a distinction can be made between the different species of the MTBC (Skuce *et al.* 1994; Aranaz, Liebana, Mateos *et al.* 1998; Michel *et al.* 2009).

1.5.3.4 IS6110- Restriction fragment length polymorphism

The insertion sequence, IS6110, is regarded as a useful marker for typing *M. tuberculosis* strains (Gutierrez *et al.* 1995) as it is present in multiple copies (ranging from 0 to 25) in different locations of the genome (van Soolingen & Arbeit 2001). The number of copies of the IS6110 element in *M. bovis* strains is usually lower (only one copy of the IS6110 element) than in *M. tuberculosis* strains (Allix, Walravens, Saegerman *et al.* 2006) and it is always present in the same location. Thus the discriminatory power of this technique is also lower when typing *M. bovis* strains. Therefore other techniques based on direct repeats (DR) (Groenen, Bunschoten, van Soolingen & van Embden 1993) are preferred for the typing of *M. bovis* isolates.

1.5.3.5 Spoligotyping

Spoligotyping (spacer oligotyping) is a PCR-based hybridization blotting typing method. In this typing practice the genetic polymorphisms are restricted to a single genomic cluster locus, the DR cluster (Roring, Scott, Brittain *et al.* 2002; Brudey, Driscoll, Rigouts *et al.* 2006). In this locus the DRs are interspersed by unique DNA spacer sequences of 35 to 41 base pairs (bp) in length (Kremer, van Soolingen, Frothingham *et al.* 1999). The presence or absence of spacers preferably at the DR locus, between different isolates is determined by spoligotyping (Kremer *et al.* 1999) and thus allows the differentiation of strains within each species belonging to the MTBC. Spoligotyping has been widely used for typing *M. bovis* isolates. It is highly reproducible, rapid, simple and produces reliable phylogenetic data (Kamerbeek *et al.* 1997).

1.5.3.6 Variable number of tandem repeats

Variable number of tandem repeats is polymorphic loci within regions of tandemly repeated DNA and were identified during the genome sequencing projects for members of the MTBC (Cole, Brosch, Parkhill *et al.* 1998). Variable number of tandem repeats typing is based upon repeat number polymorphisms within these tandemly arranged repetitive DNA sequences (Roring *et al.* 2002). These tandem repeat loci display allelic hypervariability and include exact tandem repeats A to F (Frothingham & Meeker-O'Connell 1998) but in mycobacteria the majority of the VNTRs correspond to mycobacterial interspersed repetitive units (MIRUs) (Haddad *et al.* 2004). These are mini-satellite structures composed of 51-77 bp sequences that are scattered in 41 locations throughout the bacterial chromosome (Supply, Mazara, Lesjean *et al.* 2000; Sola, Filliol, Legrand *et al.* 2003).

The VNTRs are first amplified using different pairs of primers and the products are analysed by electrophoresis or by an automated technique using primers tagged with fluorescence dye (Haddad *et al.* 2004). The use of VNTR enables exploitation for strain typing in numerous bacterial species. The availability of these different techniques has made it possible to type mycobacteria belonging to the MTBC. This is particularly useful for assisting in tracing the spread of *M. bovis* between herds and for evaluating the role of wildlife reservoirs (Haddad *et al.* 2004) in the spread and maintenance of the pathogen. These typing tools form a part of the many aspects of tackling the challenges of TB diagnostics, epidemiology and disease management. When spoligotyping is employed along with VNTRs its use is extended from

the clinical laboratory to molecular epidemiology including evolutionary and population genetics.

1.5.4 *Ante-mortem diagnosis*

Infection of mammals with mycobacteria of the MTBC results in a chronic, devastating disease (O'Reilly & Daborn 1995). Infected animals usually show no clinical signs during the early stages of the disease, which makes its diagnosis based on clinical signs challenging (Cousins 2008). For example, in cattle the clinical signs of *M. bovis* infection are not pathognomonic and infected cattle can become infectious long before they exhibit obvious clinical signs. As a result effective *ante-mortem* surveillance for the disease must primarily rely on the detection of infected cattle at an early stage by the use of sensitive immunodiagnostic tests (Cousins 2008). *Ante-mortem* diagnostic tests allow for the detection of disease during the preclinical stages of either *M. tuberculosis* or *M. bovis* infection. Tests targeting CMI (IDT, interferon-gamma (IFN- γ) assay and the lymphocyte proliferation test) and those targeting the humoral immunity (serology) have been adapted for use in domestic and wildlife species (de la Rúa-Domenech *et al.* 2006b). New developments towards the progression of reliable and cost effective *ante-mortem* diagnostic tests, in both the human (Bartalesi, Vicidomini, Goletti *et al.* 2008) and wildlife veterinary fields, are also under investigation (Lesellier, Corner, Costello *et al.* 2008; Lyashchenko, Greenwald, Esfandiari *et al.* 2008, Greenwald, Lyashchenko, Esfandiari *et al.* 2009).

1.5.5 *Immune reactions to mycobacteria*

1.5.5.1 *Cytokines*

Cytokines are a group of proteins that have the ability to regulate functions of cells and tissues, particularly immune functions. Included in this family of cytokines are, amongst many others, interferons (IFN), interleukins (IL), chemokines, colony stimulating factor (CSF) and tumour necrosis factor (TNF) (Scheerlinck & Yen 2005). According to their roles they may be subdivided into pro- and anti-inflammatory cytokines or regulatory cytokines. In the pathogenesis of TB they may be active in different phases of the disease. Of particular interest is IFN- γ . Interferon is involved in the regulation of nearly all phases of the immune and inflammatory responses (Fig. 1.1) (Schroder, Hertzog, Ravasi & Hume 2004; Alvarez 2006) and is produced by Th1 cells. Interferon- γ activates macrophages and increases

expression of the major histocompatibility complex (MHC) class I and class II molecules thus increasing antigen presentation. In its absence both humans and experimental animal hosts become susceptible to microbial infection (Rhodes, Palmer, Graham *et al.* 2000a; Pearl, Saunders, Ehlers *et al.* 2001; Widdison, Watson, Percy *et al.* 2008). Interferon- γ is considered as highly relevant for protection in the early phases of a mycobacterial infection and is the target in diagnostic assays like the IDT and IFN- γ assays.

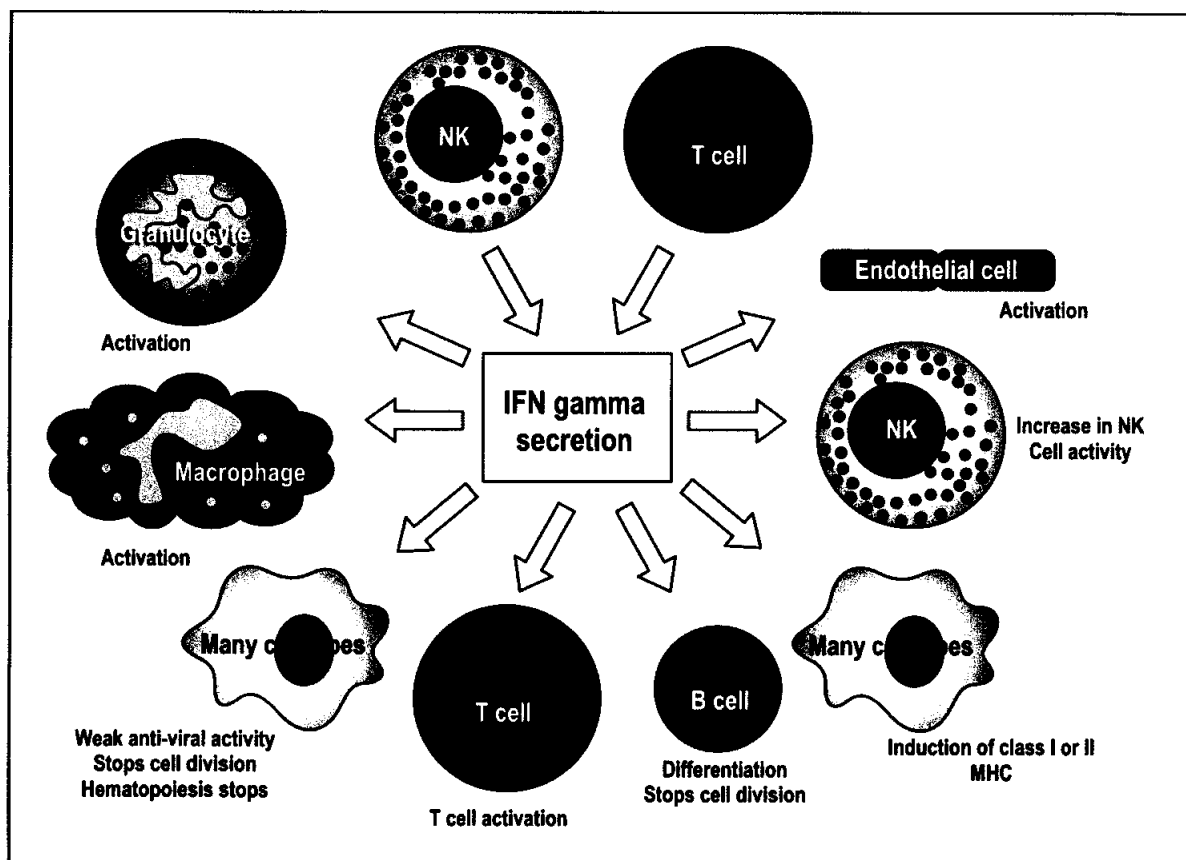


Figure 1.1 Diagrammatic representations of the pleiotropic functions of IFN- γ (Alvarez 2006)

1.5.5.2 Immunopathogenesis

When cattle are exposed to *M. bovis* various factors are produced and activated which subsequently influence the eventual outcome of infection. These factors may be inherent to the tubercle bacillus, the features of the host, and / or other factors relating to the environment (Pollock & Neill 2002). Bovine TB is primarily a pulmonary disease in cattle. The primary site of natural infection and the principal manifestation site in cattle is the respiratory tract (van Rhijn, Godfroid, Michel & Rutten 2008). Once the tubercle bacillus has entered the host

via inhalation it lodges itself within the respiratory tract in the terminal air spaces of the lungs (Pollock & Neill 2002). The host's immune response to the pathogen is influenced by the initial exposure. A high dose of infectious material will result in CMI that develops within a few weeks and a rapid production of circulating anti-*M. bovis* antibodies occurs. With lower doses there is a gradual development of CMI and little or no antibody response (Pollock, McNair, Welsh *et al.* 2001; Pollock & Neill 2002).

The preferred host cells for these pathogens are the alveolar macrophages (Glickman & Jacobs 2001). Ironically, these are key effector cells for controlling and destroying such pathogens. The pathogen is phagocytosed by macrophages and migrates in them in the lymphatic system initially to the regional lymph nodes where it may be destroyed as a result of immune responsiveness to mechanisms such as lysosomal pH, lysosomal hydrolysis, anti-bacterial peptides and superoxide (Pollock & Neill 2002; Teixeira, Abramo & Munk 2007). Mycobacteria may remain "hidden" in the phagosomes of macrophages and, in this way, are able to resist the immune systems defence mechanism. Macrophages produce IL-18, IL-12 and IL-23, and so promote Th1 cell activity. Lymphocytes release IFN- γ and TNF- α which in turn activate macrophages. This positive feedback interaction contains the infection within the T-cell-activated macrophages. It is thought that mycobacteria infect and replicate within non-activated macrophages using a variety of strategies including the avoidance of generating reactive oxygen and nitrogen intermediates, and inhibit the phagosome maturation process, thus avoiding fusion of lysosomes with the macrophage (Fairbairn, Stober, Kumararatne & Lamman 2001). This survival mechanism of mycobacteria within the macrophage can be prevented by autophagy, an intracellular pathway for the lysosomal degradation of long-lived cytoplasmic macromolecules (Harris, Master, De Haro *et al.* 2009), induced by IFN- γ . Autophagy enables infected macrophages to overcome the phagosome's maturation block, initiated by mycobacteria, and thus inhibits the intracellular survival of mycobacteria. However, on the other hand, infected macrophages release Th2 cytokines, IL-4 and IL-13, which act in inhibiting this autophagic process. Thus autophagy has the potential of producing anti-mycobacterial responses in macrophages which may be mediated by Th1 and Th2 cytokines, and shows its link with innate and adaptive immune responses against intracellular pathogens like mycobacteria (Harris *et al.* 2009).

Recognition of mycobacteria and the secretion of IL-12 by macrophages are processes initiated before the pathogen antigens are presented to T-lymphocytes. These initial stages and the production of IFN- γ in natural killer (NK) cells are induced by IL-12 (Teixeira *et al.* 2007). The primary (CMI) response helps to contain the spread of infection by induction of granuloma formation around the infective foci. Gamma-delta cells (WC1+ $\gamma\delta$) and CD2+ T-cells are present within the granulomas during the early stages (7 days after infection) of the disease (Cassidy, Bryson, Gutierrez *et al.* 2001). Granulomas progress further becoming necrotic in their centres which undergo mineralization within 10 weeks of infection, and are partially encapsulated by fibrous connective tissue. Further, a large number of lymphoid cells are present, the majority representing CD2+ T-cells and the minority WC1+ $\gamma\delta$ T-cells (Cassidy *et al.* 2001).

Gamma-delta T-cells play an important role in lesion development and might be involved in containing the bacilli through cytokine release (IL-12 and IFN- γ) and stimulating macrophage activation (McNair, Welsh & Pollock 2007). The responding T-cells also produce a range of cytokines which aid in the protective immunity against mycobacterial infections. The cytokines that are produced include IFN- γ , TNF- α , IL-4 and IL-6. T-cells (CD4+ and CD8+) also have the ability to lyse infected macrophages (Liébana, Aranaz, Aldwell *et al.* 2000). Macrophages are also involved during the early immune response (McNair *et al.* 2007). The lysis of mycobacteria by CD8+ cytotoxic T-cells is an important immune process with many of the mycobacteria killed by lytic enzymes and mycobacteria released from the macrophages are phagocytosed by more activated macrophages. If the mycobacteria causing TB are contained effectively within a bovine host, infection may become latent. It may remain in this state for years without progression but, in some, may progress to cause clinical disease. In a mouse model, results indicated that Th1-biased immune responses were predominant in ensuring bacteria remain in the latent condition and reactivation was associated with a shift towards a Th2-biased response (Pollock & Neill 2002). Th2 produces the cytokines IL-4, IL-5 and IL-10 which are involved in B-cell activation and the production of antibodies. If CMI responses fail to contain the spread of infection, i.e. mycobacteria are not killed or are not fully contained within granulomas, the animal's humoral immune system will be activated (Fig. 1.2). Since antibodies produced by activated B-cells are ineffective against mycobacteria inside macrophages, the disease will start to disseminate. Pollock, McNair,

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Welsh *et al.* (2001) reported that antibodies produced during the advanced stages of BTB are associated with the progression of the disease (Fig. 1.2).

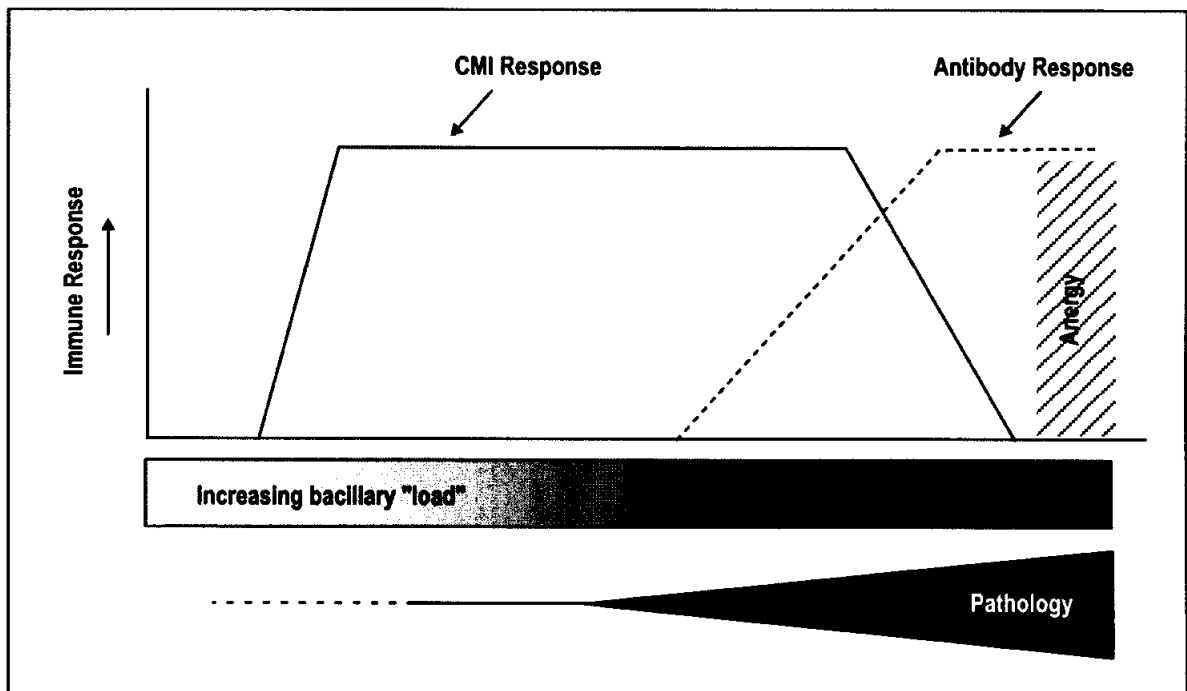


Figure 1.2 During the former (less advanced) stages of the disease the CMI response predominates. As the disease progresses CMI responses tend to wane and the humoral immunity takes over. Finally, in the clinical stage of the disease, reached by only a minority of infected animals, *M. bovis* specific immunity disappears completely, while bacterial shedding occurs (Pollock & Neill 2002)

Antibody IgG1 has been associated with lesion development and may be a useful indicator of disease status (McNair *et al.* 2007). During the progressive stage of the mycobacterial disease, which is typified by high numbers of infecting bacteria, infected animals display a lack of DTH and therefore give a negative result when tested with an IDT but have high levels of circulating antibodies. This condition is referred to as anergy (Plackett, Ripper, Corner *et al.* 1989; Neill, Cassidy, Hanna *et al.* 1994; Pollock & Neill 2002) which can be detected serologically.

1.6 Exploiting immune responses against *M. bovis* and *M. tuberculosis* for diagnosis

1.6.1 Intradermal tuberculin test

The IDT, first described by Koch (Koch 1891), is the primary method of diagnosing *M. bovis* infections in cattle and certain species of wildlife. It is based on induction of a DTH reaction,

mediated by mycobacterial antigen specific memory T-cells, upon injection of a PPD of mycobacteria into the skin. Local swelling, measured 48/72 hours later, indicates prior contact with mycobacteria (de la Rúa-Domenech *et al.* 2006b). Apart from the relevant mycobacteria, environmental mycobacteria may also induce reactivity and one has to take into account false positive results. Usually two types of tuberculin, avian PPD produced from *M. avium* (D4ER and TB56 strains) and bovine PPD produced from *M. bovis* (AN5 or Vallee strains) (Monaghan, Doherty, Collins *et al.* 1994) are used in BTB testing, but other strains may be of relevance. The skin test may be applied in one of two ways i.e. the single intradermal test (SIT), and the single intradermal comparative tuberculin test (SICTT) (Monaghan *et al.* 1994). In the former test only bovine PPD is used, whereas in the SICTT both avian and bovine PPD are injected at different sites of the animal. Using a set of interpretation criteria, a comparison is made between the extent of the reaction at injection sites to differentiate between infected animals and / or those that have been exposed to environmental mycobacteria (de la Rúa-Domenech *et al.* 2006b).

A disadvantage of BTB diagnosis using the IDT is that it requires capture of animals by immobilizing them in order to perform the test. Three days after release they have to be recaptured in order to read the results of the test. In addition, the IDT is associated with high costs and high levels of handling stress in animals due to the double immobilization (Grobler, Michel, De Klerk & Bengis 2002). Research on the production and evaluation of the use of recombinant antigens of *M. bovis* and *M. tuberculosis* is necessary to improve the sensitivity and specificity of the tuberculin test (Doherty, Monaghan, Bassett & Quinn 1995).

1.6.2 Mycobacterial antigens

Mycobacterium bovis and *M. tuberculosis* express proteins that have the ability to induce cellular immune responses in their hosts (Rhodes, Gavier-Widen, Buddle *et al.* 2000b). Among others, these include the major secreted immunogenic proteins (e.g. MPB70, MPB80, MPB83 and MPT63), early secreted antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). The soluble antigens, MPB70 and MPB80 are closely related proteins, which are expressed within the *M. bovis* “body”, whereas, MPB83 is an external lipoprotein associated with the bacterial surface (Wiker, Lyashchenko, Aksoy *et al.* 1998). The antigen, MPT63, is secreted by *M. tuberculosis* (Manca, Lyashchenko, Wiker *et al.* 1997). Early secreted antigenic target-6 is a protein secreted during the early or active stage of mycobacterial

infections (Brodin, Rosenkrands, Andersen *et al.* 2004). The ESAT-6 gene is present in *M. tuberculosis* and virulent *M. bovis* strains but absent from *M. bovis* BCG and environmental acid-fast bacteria (Harboe, Oettinger, Wiker *et al.* 1996). Both ESAT-6 and CFP-10 are potent T-cell antigens. These two antigens are used in diagnosing BTB in cattle by differentiating between non-vaccinated and BCG-vaccinated cattle (Buddle, Parlane, Keen *et al.* 1999; Vordermeier, Chambers, Cockle *et al.* 2002).

1.6.3 Blood-based tests

1.6.3.1 Serological tests

In serology, antigen-antibody complexes are measured and the results are used to aid in diagnosis of diseases of animals (Jacobson 2007). Interpretation of the results will depend largely on whether an antigen or antibody is detected. Various serological tests, such as western blots and enzyme-linked immunosorbent assays (ELISA), are available to assess the exposure of animals to pathogens, i.e. to measure the presence of antibodies. A western blot analysis is used to demonstrate the presence of antibodies against specific proteins of pathogens after their separation by electrophoresis. The ELISA is the most widely used semi-quantitative serologic assay applied for diagnosis. It is also versatile because it can be formatted in many different ways (direct, indirect and competitive) (Jacobson 2007).

For the diagnosis, in domestic animals and wildlife, serological tests have been investigated as alternatives to the IDT and other CMI-based tests. The IDT, “a *good herd test but poor animal test*”, is poor at identifying individual infected animals (de la Rua-Domenech *et al.* 2006b). Serological tests for BTB diagnosis have to be sensitive, easy to use and should be able to distinguish between different mycobacteria of the MTBC (Koo, Park, Ahn *et al.* 2005). During the initial stages of *M. bovis* infection in cattle, the cellular immunity comes into play but, as the disease progresses, humoral immune responsiveness become prominent. Therefore antibody formation is considered to be related to the advanced stages of the disease (Pollock & Neill 2002), but it should also be noted that antibodies are also present in tuberculous animals with subclinical and / or latent infections. Various antigens (MPB83, MPB70, Ag 85, ESAT-6 & CFP10) have been used in sero-diagnostic tests and it has been stated that the best option for a sero-diagnostic test for TB is to have a cocktail of antigens (Lyashchenko, Singh, Colangeli & Gennaro 2000; Amadori, Lyashchenko, Gennaro *et al.* 2002; Aagaard, Govaerts, Meikle *et al.* 2006; Liu, Guo, Wang *et al.* 2007). Koo, Park, Anh *et al.*

al. (2005) demonstrated that ELISA, latex bead agglutination assays (LBAA) and immunochromatographic assays (ICGA) using ESAT-6p, a peptide containing an immuno-dominant portion of ESAT-6, and recombinant MPB70, one of the immuno-dominant antigens of *M. bovis* and also a stable and active component of bovine PPD (Fifis, Plackett, Corner & Wood 1989), have the potential for detecting both early and advanced stages of BTB.

Recently, the Rapid Test (RT) has been developed for the diagnosis of TB in various species of animals (Drewe, Dean, Michel *et al.* 2009). Different “cocktails” of selected *M. bovis* antigens are used. This simple and rapid antibody test developed by Chembio Diagnostic Systems, Inc. employs colour latex-based lateral flow technology along with relevant TB antigens. The RT was used to quickly identify positive sera for further analysis in a multi-antigen print immunoassay (MAPIA) that utilizes a panel of 12 mycobacterial antigens which includes eight recombinant proteins (ESAT-6, CFP-10, MPB64, MPB59, MPB70, MPB83, alpha-crystallin [Acr1], a 38kDa protein), two protein fusions ([CFP-10/ESAT-6], [Acr1/MPB83BCF]), and two native antigens (bovine PPD and *M. bovis* culture filtrate [MBCF]) (Lyashchenko *et al.* 2000).

A drawback of these assays for the diagnosis of TB in wildlife species (captive as well as free-ranging) is that the numbers of positive animals available are usually low which hinders validation of these tests according to the World Organisation for Animal Health (OIE) standards. Furthermore, their use has been questioned as they show little or no reactivity that can be used for the diagnosis of pulmonary TB (Steingart *et al.* 2007). Extensive efforts to identify and characterize antigens unique to *M. bovis* for use in a diagnostic assays have shown that the antibody response to *M. bovis* is not uniform, with no evidence of a dominant persistent response to a single antigen (Cousins & Florisson 2005; Aranaz, De Juan, Bezos *et al.* 2006; de la Rua-Domenech *et al.* 2006b) at any stage of infection (Amadori *et al.* 2002; Lyashchenko, Pollock, Colangeli & Gennaro 1998). These findings suggest that some type of multiplex assay is needed to detect animals at different stages of infection (Amadori *et al.* 2002; Aagaard *et al.* 2006). To address this Whelan, Shuralev, O’Keeffe *et al.* (2008) have developed a multiplex assay that can simultaneously detect and analyse the response to multiple antigens spotted in a single well in a 96-well plate array format. The authors demonstrated the enhanced diagnostic power of a multiplex antigen approach over that of the standard-industry methods (de la Rua-Domenech *et al.* 2006b).

1.6.3.2 Lymphocyte proliferation assay

The lymphocyte proliferation assay is an *in vitro* test that measures antigen specific reactivity of memory T-cells. When using whole blood or purified peripheral blood mononuclear lymphocytes from *M. bovis*-infected cattle, proliferation induced by bovine PPD and avian PPD and can be compared for *M. bovis*-specific responsiveness and *M. avium*-related background proliferation. Griffin & Buchan (1994) made use of the lymphocyte assay to detect BTB in deer herds. The results of the initial pilot studies have shown that the proliferation assay has a sensitivity of 95% and a specificity of 92% for BTB diagnosis in infected herds. A lymphocyte proliferation assay is a time-consuming technique and restricts the number of animals that can be tested. The cells have to be incubated in complex tissue culture media for 3-5 days, and the detection of the level of cell proliferation requires the use of radioactive nucleotides. Additional disadvantages are the complicated logistics involved in the field work and needs of the laboratory. For these reasons this form of diagnosis is considered far too complex, slow and costly (Wood, Rothel, McWaters & Jones 1990; Griffin & Buchan 1994).

1.6.3.3 Interferon-gamma assay

Interferon-gamma of several veterinary relevant species of animals has been cloned for the purposes of developing effective diagnostics especially for BTB. During the last decade IFN- γ assays have especially been used in cattle to determine *M. bovis*-specific immune reactivity (Wood, Corner, Rothel *et al.* 1991; Wood & Jones 2001; Dalley, Hogarth, Hughes *et al.* 2004; Gormley, Doyle, Fitzsimons *et al.* 2006; Rhodes, Gruffydd-Jones, Gunn-Moore & Jahans 2008a; Rhodes, Gruffydd-Jones, Gunn-Moore & Jahans 2008b). The measurement of IFN- γ produced by memory T-lymphocytes after stimulation with a specific tuberculin (bovine or avian PPD as a control) assesses prior infection of an animal with *M. bovis*. The test is performed in two stages. In stage one; whole blood samples are incubated with tuberculins (bovine and avian PPD) to stimulate the lymphocytes to produce IFN- γ . After 24 hours the plasma supernatant is harvested. In stage two, the supernatants of each blood aliquot are tested for the presence of IFN- γ using a capture ELISA with a pair of bovine IFN- γ specific monoclonal antibodies (Wood & Rothel 1994). Infected animals are identified by their relative IFN- γ responses: bovine PPD versus avian PPD. Bovigam™ (Wood & Jones 2001) is the commercially available bovine IFN- γ test. In South Africa, Bovigam™ has

shown promising results for use in the diagnosis of *M. bovis* infections in African buffaloes (Grobler *et al.* 2002). As compared to the skin test, the IFN- γ test ensures minimal invasion and only a single manipulation of the animal.

1.7 Antibodies as tools to develop IGRAs

Antibodies are a class of proteins synthesized by B-cells and consist of four polypeptide chains, two heavy chains and two light chains that are connected by disulphide bonds to form a Y-shaped structure (Fig. 1.3). The variable domains V_H and V_L of heavy and light chains are unique to each antibody and together determine the antigen specificity of the molecule (Litman, Rast, Shamblott *et al.* 1993). In a regular immune response antibodies will be produced by all cells of the individual's B-cell repertoire that recognise epitopes in the antigen, i.e. polyclonal antibodies are produced. This is reflected in the immune serum. Köhler & Milstein (1975) generated populations of antibody-producing cells possessing the same specificity and affinity by fusing B-cells from immunized mice with myeloma (tumour) cells. This generated a population of immortal hybridomas, and clones producing monoclonal antibodies with the required specificity and affinity. Antibodies have been recognised as primary tools to distinguish and track target molecules, and are very important research tools (Leenaars & Hendriksen 2005). However, using hybridoma technology has proved to be very expensive and labour intensive, and maintenance of the hybridoma cultures is sometimes cumbersome. In addition, there is the welfare of experimental animals to consider.

An alternative to the polyclonal and monoclonal antibodies are recombinant antibodies produced by using phage-display technology (Smith 1985), where a single chain construct of V_H and V_L (single chain variable fragment [scFv], Fig. 1.3) is displayed on filamentous phage or released in soluble form by bacteria infected with this phage. Phage-display enables researchers to capitalize on target recognition qualities of antibodies and eventually produce large quantities of the selected clones. The recombinant antibodies have all the target recognition qualities of natural antibodies, and are produced using the same genes that code for the target recognition or variable region in natural antibodies from mammalian systems (Clackson, Hoogenboom, Griffiths & Winter 1991). Some advantages of recombinant antibodies over conventional poly- and monoclonal antibodies include libraries of a large variety of antibody gene fragments that can be cloned into expression vectors. These can, in turn, be displayed as scFvs on the surface of filamentous bacteriophage (Winter, Griffiths,

Hawkins & Hoogenboom 1994). Linking the protein function with its corresponding gene (phenotype-genotype link) is significant for the panning procedure and for accessing the gene, thus allowing for manipulations to be performed (Terpe 2003; ShiHua, JiBin, ZhiPing *et al.* 2006).

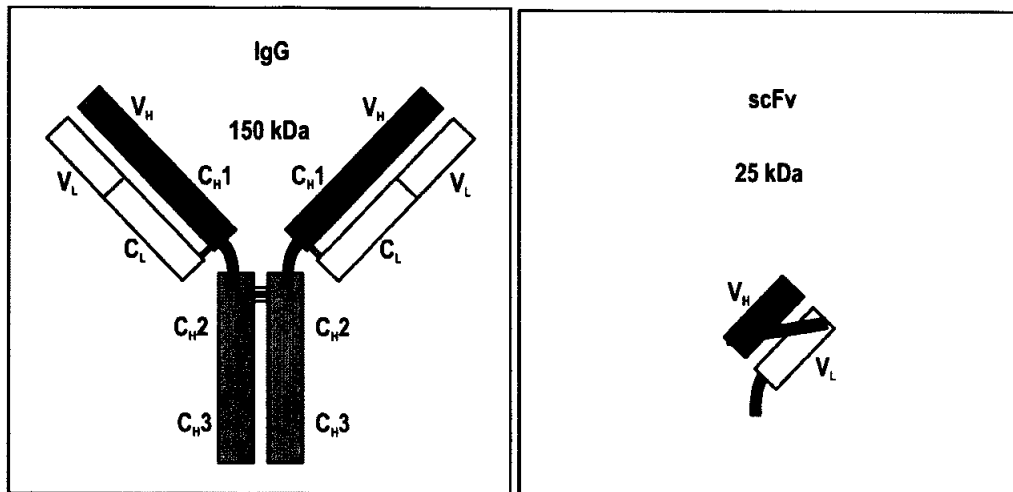


Figure 1.3 Diagrammatic representation of an IgG molecule and a single chain variable fragment

Phage libraries can be constructed from either naïve or immune libraries. The difference between the two is that the former are derived from unimmunized, rearranged, synthetic or shuffled V-genes, the last named being created from V-genes from immunized animals or humans. The immune library can have a strong bias towards antibodies of certain specificity but can also be used to select antibodies against antigens which have not been used in the immunization (Bradbury & Marks 2004). Selection of the desired clones is performed by a technique called bio-panning (Fig. 1.4), which involves the selection of specific binders from phage libraries using the protein of choice (Russel, Lowman & Clackson 1989; Clackson & Wells 1994). Briefly, the target antigen is immobilized in an immunotube. The phages which display the scFvs, bind to the antigen and unbound phages are washed away. Bound phages are eluted, amplified in *Escherichia coli* and then subjected to further rounds of panning. This should result in the enrichment of the number of phages binding to the antigen. Clones from the final round (after Round 3-4 of selection) are characterized individually. Characterization is done by ELISA, which is then followed by sequencing the DNA inserts of the reacting

clones. Once the sequences of the selected clones are known, they can be tailored according to test demands.

Chickens have been used to generate recombinant antibodies (Yamanaka, Inoue & Ikeda-Tanaka 1996; Sapats, Trinidad, Gould *et al.* 2006; Hof, Hoeke & Raats 2008). These animals diversify their immune responses by using only one recombination event in combination with a process called 'gene conversion' (Reynaud, Anquez, Grimal & Weill 1987). During this process, pseudogenes are translocated into the heavy and light chain variable regions; as a result only two primer sets are needed to construct chicken libraries (Davies, Smith, Birkett *et al.* 1995). Therefore chicken antibody libraries are easier and faster to construct compared to human and murine libraries (Hof *et al.* 2008). Chickens are more likely to evoke an immune response to epitopes that are highly conserved between mammals. Furthermore, chicken antibodies have low cross-reactivity to some proteins and display high specificity compared to other IgG antibodies (Gassmann, Thommes, Weiser & Hubscher 1990).

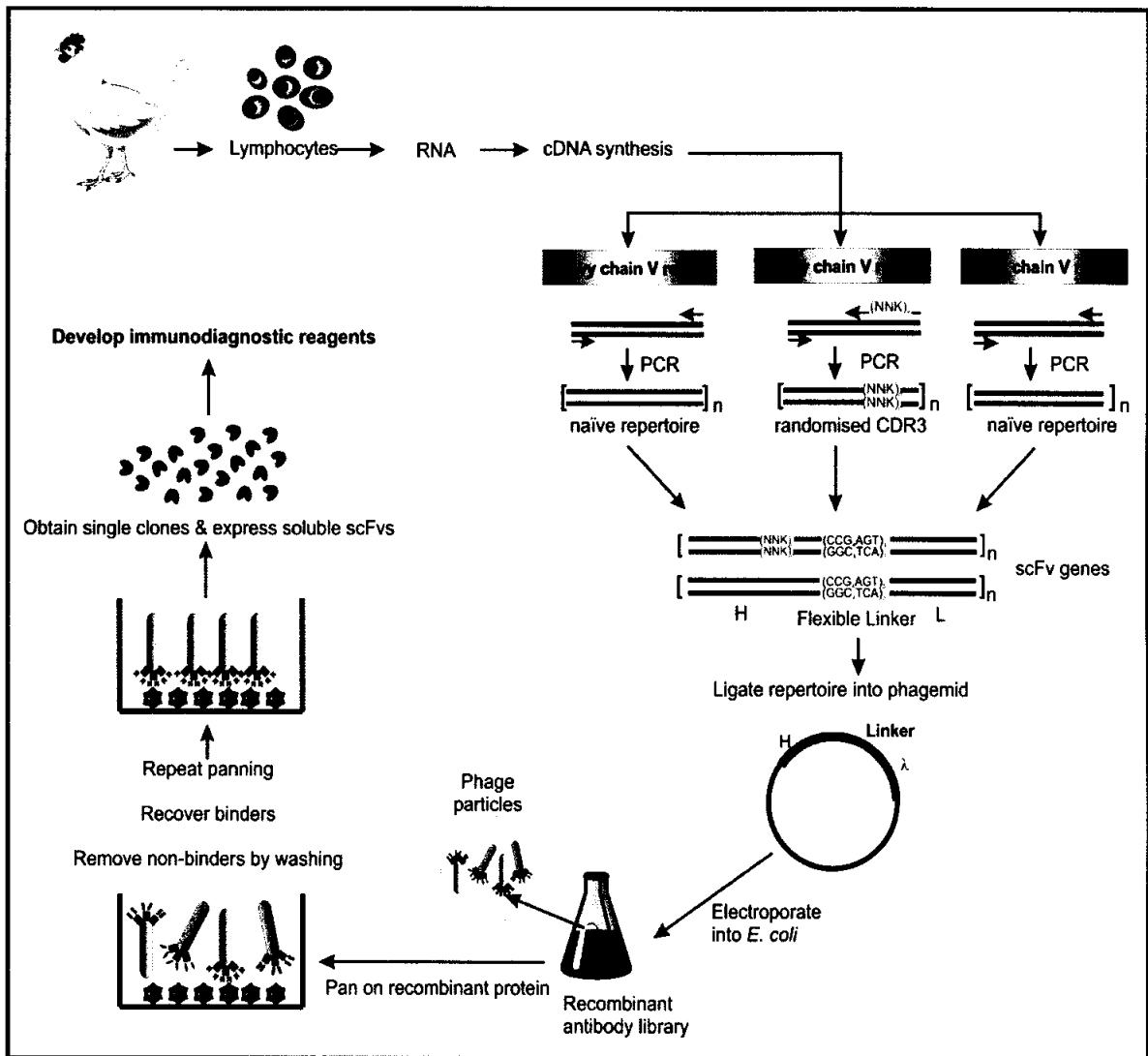


Figure 1.4 Construction of a phage-display antibody library from an immunized chicken. The diagram shows the assembly of the scFv using a three fragment assembly reaction. The construct is cloned into a vector and electroporated into electro-competent *E. coli* cells. Phage particles are then selected against an immobilized antigen by bio-panning. After repeated panning rounds single clones expressing scFv are obtained (Fehrsen, van Wyngaardt, Mashau *et al.* 2005)

1.8 Problem statement

In recent decades TB caused by *M. bovis* has spread from cattle, the primary hosts, to captive and free-ranging wildlife and may affect humans. Similarly, *M. tuberculosis* has been shown to infect domestic and wildlife species, predominantly wildlife in captivity but recently also in some that are free-ranging. At the wildlife-livestock-human interface transmission between species may occur continuously. In South African wildlife the African buffalo and,

potentially, the kudu, are the maintenance hosts of BTB. Spill-over of infection has been reported in lions, cheetahs, leopards, baboons, warthogs and honey badgers. In addition, the white rhinoceros (near threatened species) and black rhinoceros (critically endangered species) (www.iucnredlist.org) have been identified to be susceptible to both *M. bovis* (Dalovisio *et al.* 1992) and *M. tuberculosis* (Oh *et al.* 2002) infections based on culture results. The emergence of *M. tuberculosis* infection in domesticated and captive Asian elephants in the USA, Europe and Asian countries also causes major concern regarding the sensitivity of free-ranging animals.

Wildlife is one of South Africa's biggest assets from an economic standpoint, and a chronic debilitating disease which directly affects a wide spectrum of wildlife species may result in the collapse of conservation programmes. An outbreak of *M. bovis* / *M. tuberculosis* infections in domesticated, captive and free-ranging wildlife populations poses a significant health threat to wildlife populations in conservation areas, livestock and people in neighbouring communities and livestock farming communities that border game reserves (Michel 2002). The problem that wildlife conservationists, researchers and veterinarians are currently faced with is that there is no single test available for use in wildlife that can diagnose the infection with high specificity and sensitivity prior to the onset of clinical signs and before the animal becomes contagious (i.e. a shedder) for its congeners. Diagnostic tools are not validated or are not available for most wildlife species. There is a registered vaccine for humans (BCG) but there is no registered vaccine for animals. These limitations make diagnosis, control, management and eradication of the disease, especially in free-ranging ecosystems, very challenging.

At present there is no proof that *M. bovis* infections have spread to species such as black and white rhinoceroses, and African elephants in wildlife sanctuaries in South Africa. The possibility of such an occurrence in the future cannot be ruled out, and the full effect of the present epidemic, occurring in some game reserves, may only be seen in the long term. These negative effects threaten the biodiversity of wildlife and the survival of threatened or endangered species as well as programmes for their relocation and trade.

The diagnosis of BTB is a complex, multifaceted problem in the context of the wildlife-livestock interface. Thus attempting to solve the BTB epidemic in wildlife requires an accurate understanding of its magnitude and extent of spread within a geographical area. As

for human TB, the choice of a diagnostic test depends upon the setting in which the test is to be performed and the intended use of the results (fit for purpose principle). In species in which *M. bovis* infections are enzootic, such as the buffalo populations in the KNP and HiP, surveys will provide data on the spatio-temporal distribution as well as prevalence rate estimates of the disease. However, in order to provide the parks authorities with management tools, the ideal diagnostic tool should identify the shedders so that the disease can be managed by culling such animals. In this case, a diagnostic test identifying the active disease is required and it should, in addition, be highly specific.

In species, such as domesticated and captive Asian elephants, known to be infected by *M. tuberculosis*, the identification of shedders is even more important, given the risk for humans contracting TB from these animals. In species currently free from *M. bovis* / *M. tuberculosis* infections (e.g. the African elephant, and black and white rhinoceroses), the ideal diagnostic tool should identify infected animals as early as possible, before an animal becomes contagious or shows clinical signs. Such animals should be quarantined and treated or euthanased to maintain a BTB-free population. In this case, the first test to be used should be highly sensitive. Once the risk animal or animals are identified a rigorous diagnostic work-up can be performed. A further challenge lies in the fact that diagnosis has to be performed in a wide diversity of species susceptible to the disease. Many tests exist for BTB diagnostics but they all suffer from one or another shortcoming when applied in the wildlife context. For example, the IDT which is the standard method of detecting *M. bovis* infections in domestic cattle is not validated in many wildlife species. Other options that have been pursued to overcome limitations associated with skin tests include serology-based assays. However, to date, no serology-based assay has shown the sensitivity and / or specificity that are required for a routine diagnostic tool. Since immune responses (including protective immunity) against mycobacteria are primarily CMI-based, diagnostic tests measuring CMI are considered to be the most important diagnostic tools for the identification of animals infected with *M. bovis* or *M. tuberculosis* (Pollock *et al.* 2001; Pollock, Welsh & McNair 2005) prior to the onset of clinical signs. Especially the IFN- γ tests, applied in some instances for the diagnosis of BTB in cattle and which, most likely, will be in the near future, the method of choice in diagnosis of TB in humans, have to be developed for relevant species. Moreover, IFN- γ tests may be performed by using different antigens. Some of these antigens are considered to be “early” antigens, for example MPB70 in cattle, while others are considered

to be correlated to active TB in humans or BTB in cattle (e.g. ESAT6 and CFP10) (Vordermeier *et al.* 2002; Jafari & Lange 2008).

1.9 Research objectives

The aim of the study was to develop IFN- γ capture ELISAs for the detection of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants. To achieve this goal, the reagents of the ELISA system had to be developed. The first objective was to clone, sequence and express the IFN- γ gene of the white rhinoceros and Asian elephant. This was followed by the sequencing of the AfEpIFN- γ gene and the investigation of the similarities between this gene and the AsEpIFN- γ gene. The second objective was to produce anti-rRhIFN- γ monoclonal and polyclonal antibodies in mice and chickens respectively. The third objective was to use the phage-display technique to generate recombinant antibodies against recombinant white rhinoceros and AsEpIFN- γ derived from chickens and the fourth objective was to produce anti-rAsEpIFN- γ monoclonal antibodies in mice. Antibodies that were produced and characterized during these studies were utilized in different combinations for the development of rhinoceros and elephant (recombinant) IFN- γ ELISAs.

1.10 Impact

The availability of IFN- γ detection systems for the diagnosis of *M. bovis* or *M. tuberculosis* infections in valuable wildlife species would contribute towards the efforts of controlling the infection, and will prove to be a valuable tool for relocation and breeding strategies. Furthermore, they would enable refinement of testing by the use of different recombinant antigens instead of rather crude PPD preparations according to the goal to be pursued i.e. early detection as compared to identification of shedders.

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Chapter 2

Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) interferon-gamma and the production of white rhinoceros interferon-gamma specific antibodies

Abstract

Bovine tuberculosis (BTB) is endemic in African buffaloes (*Syncerus caffer*) in the Kruger National Park (KNP). In addition to buffaloes, *Mycobacterium bovis* has been found in at least 14 other mammalian species in South Africa, including kudu (*Tragelaphus strepsiceros*), chacma baboons (*Papio ursinus*) and lions (*Panthera leo*). This has raised concern about the spillover into other potentially susceptible species such rhinoceroses, thus jeopardising breeding and relocation projects aiming at the conservation of biodiversity. Hence procedures to screen for and diagnose BTB in the black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) need to be in place. The interferon-gamma (IFN- γ) assay is used as a routine diagnostic tool to determine infection of cattle and, recently, African buffaloes, with *M. bovis* and other mycobacteria. The aim of this study was to develop reagents to set up a rhinoceros IFN- γ (RhIFN- γ) assay. The white RhIFN- γ gene was cloned, sequenced and expressed as a mature protein. Amino acid sequence analysis revealed that RhIFN- γ shares a homology of 90% with equine IFN- γ . Monoclonal antibodies, as well as polyclonal chicken antibodies (Yolk Immunoglobulin-IgY) with specificity for recombinant RhIFN- γ , were produced. Using monoclonal 1H11 as a capture antibody and polyclonal IgY for detection, it was shown that recombinant as well as native white RhIFN- γ was recognised. This preliminary IFN- γ enzyme-linked immunosorbent assay has the potential to be developed into a diagnostic assay for *M. bovis* infection in rhinoceroses.

2.1 Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) and has mostly presented itself as a problem in cattle (O'Reilly & Daborn 1995; Pollock, Rodgers, Welsh & McNair 2006) and related species including goats (Cousins, Francis, Casey & Mayberry 1993; Liébana, Aranaz, Urquia *et al.* 1998) worldwide. More recently *M. bovis* has also been found to infect wildlife species such as the lion (*Panthera leo*), kudu (*Tragelaphus strepsiceros*), African buffalo (*Syncerus caffer*) and chacma baboon (*Papio ursinus*) in South Africa (Michel, Bengis, Keet *et al.* 2006), European badger (*Meles meles*) (Cheeseman, Wilesmith & Stuart 1989) in the United Kingdom and the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Buddle, Skinner & Chambers 2000). In the Kruger National Park (KNP) and elsewhere, potential spillover into other species, such as the rhinoceros, for which currently no validated *ante-mortem* (indirect) diagnostic tools exist, may jeopardise breeding and relocation projects aimed towards the conservation of biodiversity. The development of practical and reliable procedures to diagnose BTB in black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses have therefore been identified as a priority area for research by conservation bodies.

Although BTB has to date not been diagnosed in pachyderms in South Africa, it is of utmost importance to be able to provide an additional guarantee on the TB-free status of these animals and to provide conservation bodies with an early warning system should BTB enter the rhinoceros population. While tests traditionally available for diagnosing BTB include microscopic and bacterial culture techniques, as well as tuberculin skin tests (TST) (Wood, Rothel, McWaters & Jones 1990b; Monaghan, Doherty, Collins *et al.* 1994), these are of little value for screening purposes in pachyderms. Culture techniques are the most reliable and specific, but have the drawback that they generally require post-mortem specimens, and results are obtained only after 6 to 8 weeks. Specificity and sensitivity of TST have been determined mostly for domestic animals, especially cattle (Monaghan *et al.* 1994), but diagnosing *M. bovis* infection in wildlife is proving to be a challenge.

In pachyderms, such as the rhinoceros and elephant, TST are not practical both due to difficulties in defining suitable injection sites and the fact that these reactions have to be read after approximately 72 hours, which necessitates the recapture of the animals. As an alternative to TST, interferon-gamma (IFN- γ) assays, for example the Bovigam™ test

(Wood, Corner & Plackett 1990a; Wood *et al.* 1990b; Wood & Jones 2001), have been used during the last decade to determine *M. bovis*-specific immune responses in ruminants. These tests consist of antibody-based capture enzyme-linked immunosorbent assays (ELISA) that will detect IFN- γ produced by specific T-cells after incubation of heparinized blood with *M. bovis* antigens. Infected animals are identified by their high IFN- γ responses, due to *M. bovis* antigen specific T-cells induced by the mycobacterial infection.

In this chapter, the first steps in developing an IFN- γ -based capture ELISA for the detection of *M. bovis* infection in the white rhinoceros are described. Furthermore, the first evaluation of the specificity of the test in known TB-free rhinoceroses is reported. The TB-free status of these white rhinoceroses was determined based mainly on the epidemiological evidence of the absence of an *M. bovis* infection in rhinoceroses in South Africa. Indeed to date, after more than 10 years of translocation programmes, no single case of *M. bovis*- or *M. tuberculosis*-infected rhinoceros has been documented (Michel *et al.* 2006).

The set up of the test includes the cloning, sequencing and the expression of the white rhinoceros IFN- γ (RhIFN- γ) gene and the production of RhIFN- γ specific monoclonal and polyclonal antibodies. Thus, the IFN- γ produced *in vitro* by antigen stimulation of sensitized T-lymphocytes can be measured to serve as a sensitive and specific indicator of *M. bovis* exposure. Development of this assay could ultimately yield a vital tool for detecting *M. bovis* infection in rhinoceroses prior to the development of clinical signs.

2.2 Materials and Methods

2.2.1 Cloning and sequencing of white RhIFN- γ

Blood from an adult white rhinoceros was collected in ethylenediaminetetraacetic acid (EDTA) Vacutainer™ tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Amersham 17-1440-02). After 25 min of centrifugation at 2 800 rpm, mononuclear cells were taken from the interphase and washed twice in RPMI-1640 medium supplemented with L-glutamine (Sigma, R8758) and 10% heat inactivated foetal calf serum (FCS). To induce IFN- γ production, purified mononuclear cells (1×10^6 cells/ml in wells of a 24-well plate) were stimulated with 5 μ g/ml

concanavalin A (Con A) (Sigma, C2010-100mg) for 18 to 24 h at 37°C in a 5% CO₂ incubator.

Total RNA was purified from stimulated lymphocytes using Trizol reagent (Gibco BRL, Life Technologies, 15596-018) and 1 µg of total RNA was subjected to first strand cDNA synthesis using reverse transcriptase Rnase H SuperscriptII™ (Gibco BRL, Life Technologies, 18064-014) and oligo-(dT)₁₂₋₁₈ as a primer for RT-PCR. The cDNA produced in this way was used as a template for a polymerase chain reaction (PCR) using *Pwo* DNA polymerase (Roche, 1644 947) according to the manufacturer's instructions. PCR primers (5'-end primer sequence: 5'-GCCGCGCGGGAGCCAGGCCGCGTTTTTTTAAAGAAATAG-3' and 3'-end primer sequence: 5'-GCGGCGGCGGGAATTCAAATATTGCAGGCAGG-3') used were designed to amplify the part of the white RhIFN-γ gene that encodes the mature protein (without signal sequence). The sequence of the primers was based on the equine IFN-γ (EqIFN-γ) gene (Genbank Accession Number - D28520), because this species has a close phylogenetic relationship with rhinoceroses.

A gradient PCR was performed for 35 cycles using a BioRad Thermal iCycler. One cycle consisted of: DNA denaturation at 95°C for 30 s, primer annealing at 56°C, 58°C, 61°C or 64.7°C (depending on the position of the PCR tube in the gradient) for 45 s and primer extension at 72°C for 60 s. The underlined sequences in the primers above are not part of the IFN-γ sequence, but were included as annealing sites for a second PCR performed with the forward primer GW2- F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGTGCCGCGCGGGAGC-3') and the reverse primer GW-R (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGCGGCGGCGGG-3'). This second PCR, introduced the *attB1* and *attB2* sites, which enabled subsequent Gateway® (GW) cloning (Invitrogen). The conditions for this second PCR were similar to those described for the first PCR, apart from the annealing temperature that was set at 56 °C. Following DNA electrophoresis, the PCR product was harvested from the low melting point agarose gel and inserted into the vector pDONR201 (Invitrogen) by the BP GW reaction (Invitrogen – Gateway™ BP Enzyme Mix, 11789-013) performed according to the manufacturer's instructions.

After transformation of *Escherichia coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to check the cloned fragment. Subsequently, the IFN-γ gene

was subcloned into the expression vector pET15bGW by the LR GW reaction (Invitrogen, Life Technologies - Gateway™ LR Clonase Mix, 11791-091). The resulting expression vector was designated pET15-RhIFN- γ . Vector pET15bGW is a derivative of pET15b (Novagen) that was adapted for GW cloning (Invitrogen) by ligation of the GW cassette containing *XbaI-HindIII* fragment of pDEST17 (Invitrogen) into the corresponding sites of pET15b. The resulting plasmid was purified from liquid culture from ampicillin- and chloroamphenicol-resistant colonies obtained after transformation of *E. coli* DB3.1 (Invitrogen).

To determine the 5'-end of the complete coding part of the RhIFN- γ -gene (including the sequence encoding the signal sequence), this end was cloned separately. First, it was amplified under the PCR using *KOD* hotstart polymerase (Novogen) according to the manufacturer's instructions. The white rhinoceros cDNA described above was used as a template. The forward primer F3 (3'-CCTGATCAGCTTAGTACAGAAGTGA-5') was based on the published EqIFN- γ sequence upstream of the start codon and the reverse primer R3 (5'-TCCTCTTTCCAGTTCTTCAAGATATC-3') based on the RhIFN- γ gene sequence encoding the mature protein that had been cloned as described above. A gradient PCR, with annealing temperatures between 65°C and 55°C, resulted in a weak PCR band of the expected size for the reaction performed at 57°C. To obtain enough material for cloning, additional PCR rounds were required. To avoid amplification of the smear of unspecific PCR bands present, a half-nested PCR was performed on the original PCR product to increase the specificity. For this half-nested PCR, the same forward primer (F3) was used, because the 5'-end sequence was not known, and a reverse primer R7 (5'-TCATTCATCACTTTGATGAGTTCA-3') which anneals to a sequence upstream of primer R3 (internal reverse primer) was used. For the first 20 cycles, the annealing temperature was reduced by 0.5°C for each cycle (touch down PCR), starting at 65°C. The following 20 cycles were performed at a constant annealing temperature of 55°C. The resulting PCR product was analysed on a 1.5% low melting point agarose gel and a dominant PCR band of the expected size (approximately 450 bps) was cut out. After melting the agarose at 65°C, this PCR band was used as a template for another half-nested PCR, using the same forward primer as a reverse primer R4 (5'-CCTCTTTCCAGTTCTTCAAGATATC-3') which anneals to a sequence upstream of primer R7. The PCR conditions were identical to those described for the previous half-nested PCR, except that only ten cycles were performed, once the touch

down PCR reached an annealing temperature of 55°C. The obtained PCR product was cloned into the vector pCR4, using the “Zero-blunt-TOPO-PCR-cloning-kit” (Invitrogen) according to the manufacturer’s instructions. After transformation of *E. coli* strain DH5 α , plasmid DNA was purified from selected colonies and sequenced to verify the cloned fragment. The successive rounds of this half-nested PCR approach were performed to obtain enough material to allow efficient cloning and to increase the specificity.

2.2.2 Expression and purification of recombinant white RhIFN- γ

Vector pET15-RhIFN- γ was used to transform *E. coli* BL21-codon⁺® (DE3)-RIL competent cells (Stratagene, 230245). A single ampicillin- and chloramphenicol-resistant colony was spread on a Luria Broth (LB) agar plate containing ampicillin, chloramphenicol and 1% (w/v) glucose. The glucose was added to repress expression of the recombinant protein. After overnight incubation at 37°C, the bacteria were harvested from the plate with an inoculation loop and resuspended in 10 ml LB medium. After resuspension the bacteria were transferred to 500 ml LB medium containing ampicillin and chloramphenicol and incubated at 37°C, with shaking, until the optical density at 600 nm (OD₆₀₀) reached 0.6-0.9. Gene expression was induced with 1 mM isopropylthio- β -D-galactosidase (IPTG) and incubation continued for 4 h, after which cells were harvested by centrifugation at 5 000xg for 15 min. Cell pellets were resuspended in 40 ml of Buffer B (20 mM Tris HCl pH8, 500 mM NaCl) containing 0.1 mg/ml of lysozyme. The cell suspension was transferred to a 50 ml tube and was incubated at room temperature under rotation for 30 min. This was followed by addition of 5 ml Buffer C (100 mM DTT, 50 mM EDTA, 10% Triton X 100). The contents of the tube were mixed by inverting the tubes several times and the lysate was prepared by repeating (three times) alternate freezing and thawing steps at -20°C and room temperature respectively. After the last freeze / thaw step, 1 500 units of benzonase (Novagen) and 1.5 ml of a 0.5 M MgCl₂ solution were added and the mixture incubated at room temperature for 30 min to break down the DNA and reduce the viscosity. The total protein lysate was centrifuged at 5 000xg for 15 min. The pellet, containing the IFN- γ inclusion bodies, was washed with Buffer B containing 1% Triton X 100. After a final centrifugation step (5 000xg for 15 min) the pellet was dissolved in 10 M urea prepared freshly in Buffer B containing 20 mM imidazole at room temperature. Any remaining insoluble material was removed by centrifugation at 5 000xg for 20 min at room temperature. The hexa-histidine tagged recombinant IFN- γ was purified by immobilized metal affinity chromatography (IMAC) on chelating sepharose fast flow

(Amersham-Biosciences, 17-0575-02) charged with Ni²⁺ according to the manufacturer's instructions. After equilibration of the column with Buffer B containing 20 mM imidazole and 8 M urea, the solved inclusion bodies were applied to the column. The bound protein was washed with ten column volumes of Buffer B containing 20 mM imidazole and 8 M urea. The protein was refolded on the column by fast replacement (rapid switching) of wash buffer with two column volumes of refolding buffer (50 mM Tris HCl pH8, 2 mM oxidized glutathione, 0.22 mM reduced glutathione, 1 M NDSB201 [Merck], 0.5 M L-Arginine) and incubated at 4°C for 40 h. Refolding buffer was discarded and the column washed twice with one column volume PBS containing 1 M NDSB201. The refolded IFN- γ was eluted with a total of five column volumes of PBS containing 50 mM EDTA. The protein was dialysed against 1 X PBS and subsequently centrifuged (5 000xg for 30 min) to remove any protein that had precipitated during the dialysis. The protein solution was mixed with an equal volume of glycerine and then sterile-filtered using a 0.2 μ M filter and stored at -20°C. Samples were taken during the whole purification process for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.3 *White RhIFN- γ specific poly- and monoclonal antibodies*

Mice were immunized with recombinant RhIFN- γ (rRhIFN- γ) according to routine procedures using Specol, an oil-based adjuvant (Bokhout, van Gaalen & van der Heijden 1981; Hall, Molitor, Joo & Pijoan 1989; Boersma, Bogaerts, Bianchi & Claassen 1992) and boosted on three occasions. When desired serum antibody titres were achieved, as determined by direct rRhIFN- γ ELISA, mice spleen cells were fused to SP2/0 cells to obtain hybridomas and plated in 96-well tissue culture plates (Köhler & Milstein 1975). Supernatants from wells containing colonies of hybridoma cells were tested in the ELISA for the presence of rRhIFN- γ specific monoclonal antibodies. Positive colonies were subcloned by FACS Vantage (Becton Dickson) single cell sorting, based on forward / sideward scatter characteristics, and tested again.

For the production of polyclonal antibodies, chickens were immunized intramuscularly using Specol as an adjuvant, and boosted on a regular basis to maintain antibody titres. Eggs were collected for up to 1 year following the first booster immunization and stored at 4°C till further processing. Finally, antibodies were purified from the egg yolk by the "water dilution method" followed by ammonium sulphate precipitation according to the procedure described

by Hansen, Scoble, Hanson & Hoogenraad (1998). After extensive dialysis against PBS, poly- and monoclonal antibodies were sterile filtered using a 0.2 μM filter, aliquoted and stored at 4°C until use. The immunization protocols were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

2.2.4 Screening of hybridomas for antibody production by indirect ELISA

Fifty microlitres per well of the rRhIFN- γ protein diluted to 1 $\mu\text{g}/\text{ml}$ in carbonate buffer (0.1 M, pH9.6) were used to coat 96-well Costar high binding ELISA plates overnight at 4°C. After removal of the coating buffer, the plates were blocked with 100 $\mu\text{l}/\text{well}$ of 2% fat-free milk powder (Protifar) in PBS for 1 h at 37°C. Plates were then washed five times using tap water with 0.1% Tween 20 using an ELISA plate washer. Fifty microlitres of hybridoma supernatants diluted 1:1 with 2% Protifar in PBS containing 0.1% Tween 20, were added to the wells. After 1 h at 37°C and five additional washings, goat anti-mouse IgG (1:2000) horse radish peroxidase (HRP) conjugate (Boehinger Mannheim, 1047523) in Protifar in PBS containing 0.1% Tween 20 was added and plates were incubated for 1 h at 37°C. After five additional washes, ABTS (5 mg/ml) (Roche, 1112597) was used as the substrate for the colour reaction. After 30 min at room temperature plates were read spectrophotometrically (BioRad) at 492 nm.

2.2.5 Native IFN- γ

Blood was collected in EDTA Vacutainer™ tubes from three TB-free white rhinoceroses. Purified PBMCs were isolated as described above. To induce IFN- γ production, purified PBMCs (1 X 10⁶ cells/ml in wells of a 24-well plate) were stimulated with 10 $\mu\text{g}/\text{ml}$ Con A (Sigma, C2010-100mg) at 37°C in a 5% CO₂ incubator. A control sample was included consisting of PBMC cultured without mitogen. After 18 to 24 h incubation, cell cultures were collected and centrifuged at 3 200 rpm for 10 min and the supernatant harvested. Production of IFN- γ was analysed in the capture ELISA described below.

2.2.6 Prototype capture ELISA for detection of native white RhIFN- γ

Microwell™ polysorb ELISA plates (Nunc, C96 446140) were coated with 50 μl of monoclonal antibody 1H11 at 1 $\mu\text{g}/\text{ml}$ and incubated overnight at 4°C. Wells were blocked

with 100 μ l block buffer (Protifar in PBS) and incubated at 37°C for 1 h. The plates were washed with wash buffer (H₂O/0.1% Tween 20) five times. As a positive control white rRhIFN- γ was diluted in PBS to 1 μ g/ml and tested in duplicate. Undiluted supernatants (50 μ l) collected from overnight stimulated PBMCs were added to the remainder of the wells. After the incubation the wells were washed five times with wash buffer and incubated with 50 μ l polyclonal antibodies to white RhIFN- γ (chicken IgY 700 μ g/ml, 1:100 dilution in block buffer) per well. After 1 h the plates were washed five times with wash buffer and rabbit polyclonal to chicken IgY H&L (HRP) (Abcam, ab6753) antibody was added (1:3000 dilution). The wash step was repeated and the addition of *ortho*-phenylenediamine (OPD) (Sigma, P3804) substrate followed. The reaction was stopped after 20 min with 50 μ l of 2 N H₂SO₄ and the absorbance was read 10 min later at 492 nm.

2.3 Results

2.3.1 Cloning and sequencing of the white RhIFN- γ gene

Initially the part of the IFN- γ gene encoding the mature protein was amplified by reverse transcriptase-PCR (RT-PCR) using primers that were based on the EqIFN- γ sequence. A single PCR band was obtained (results not shown) that was cloned into the GW vector pDONR201. Sequencing demonstrated strong homology of the cloned PCR fragment with the EqIFN- γ gene. The RhIFN- γ gene was subsequently cloned into an *E. coli* expression vector (pET15b-GW). To determine the total coding sequence of the RhIFN- γ gene, the missing 5'-end was cloned separately, using a forward primer that was based on the equine 5'-end IFN- γ sequence and reverse primers based on the cloned sequence encoding the mature part of white RhIFN- γ . The complete coding sequence was composed of this 5'-end sequence and the previously determined sequence encoding the mature IFN- γ . The nucleotide (nt) and predicted amino acid (aa) sequences of white RhIFN- γ are shown in Fig. 2.1 and 2.2 respectively. The coding part of the white RhIFN- γ gene is 501 nucleotides long and encodes a protein with a predicted molecular weight (MW) of 19.4 kDa. According to the SignalP 3.0 prediction server (www.cbs.dtu.dk/services/SignalP/) the most likely signal peptidase cleavage site is located between aa 25 and 26, which would yield a mature protein of 141 aa and a signal peptide of 25 aa. The predicted amino acid sequences of white RhIFN- γ and EqIFN- γ (Grunig, Himmler & Antczak 1994) are aligned in Fig. 2.2.

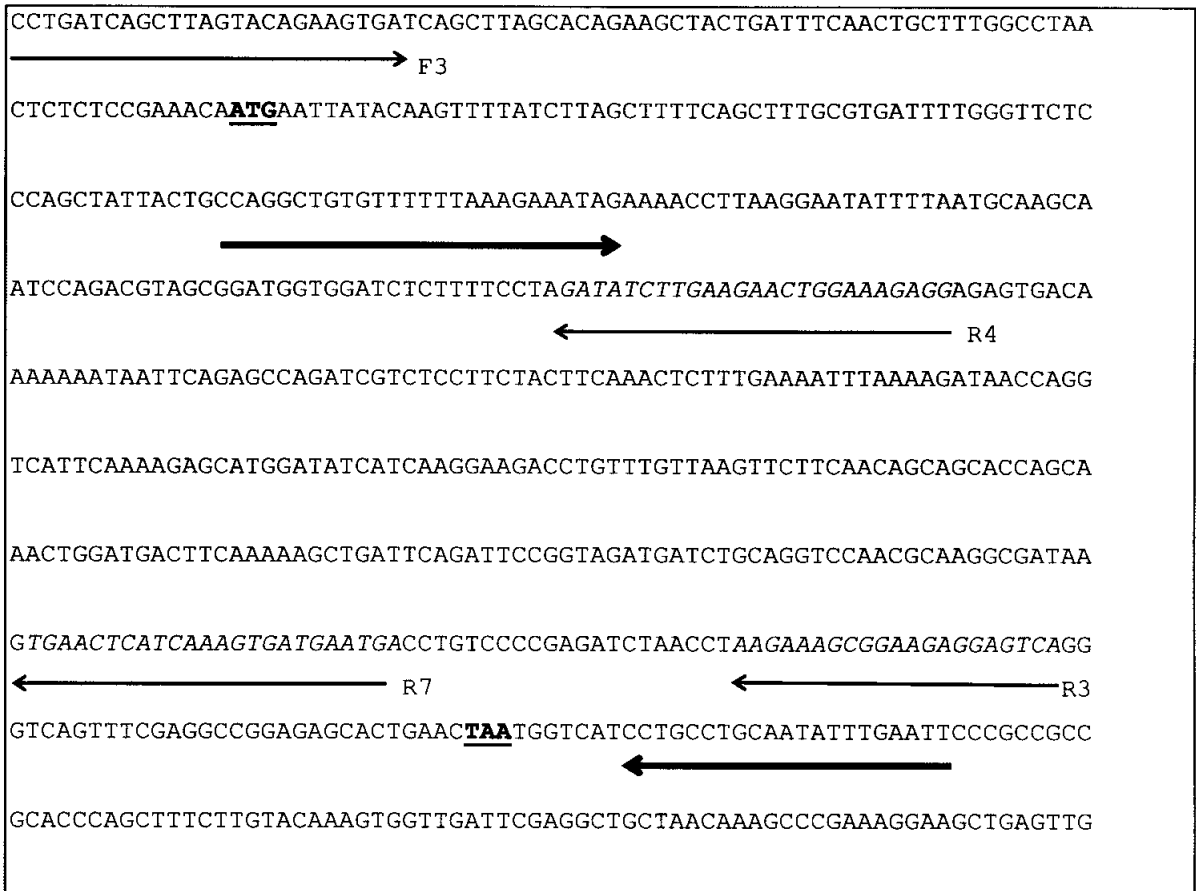


Figure 2.1 Nucleotide sequence of RhIFN- γ gene (Genbank Accession Number: DQ305037). Bold arrows indicate annealing regions used by PCR primers for cloning the sequence encoding the mature protein. Primers employed for determining the 5'-end of the gene are indicated with thin arrows. The start codon (ATG) and the stop codon (TAA) are in bold and underlined

Rhino	<u>MNYTSFILAFQLCVILGSPSYCQAVFFKEIENLKEYFNASNPDVADGGSLFLDILKNWK</u> 60
	MNYTSFILAFQLC ILGS +YYCQA FFKEIENLKEYFNASNPDV DGG LFLDILKNWK
Horse	MNYTSFILAFQLCAILGSSTYYCQAFFKEIENLKEYFNASNPDVGDGGPLFLDILKNWK 60
Rhino	EESDKKIIQSQIVSFYFKLFENLNQVIQKSMDIKEDLFVKFFNSSTSKLDDFKKLIQ 120
	E+SDKKIIQSQIVSFYFKLFENLNQVIQKSM DIKEDLFVKFFNSSTSKL+DF+KLIQ
Horse	EDSDKKIIQSQIVSFYFKLFENLNQVIQKSM DTIKEDLFVKFFNSSTSKLEDFQKLIQ 120
Rhino:	IPVDDLQVQRKAISELIKVMNDLSPRSNLRKRKRSQGFRRALN 166
	IPV+DL+VQRKAISELIKVMNDLSP++NLRKRKRSQ FRGRRAL
Horse	IPVNDLKVQRKAISELIKVMNDLSPKANLRKRKRSQNPFRGRRALQ 166

Figure 2.2 Alignment of predicted protein sequences for white RhIFN- γ and EqIFN- γ . Amino acid identities are shown in blue and a plus sign denotes conserved substitution. The predicted signal sequence of RhIFN- γ is underlined. A blast search of the white RhIFN- γ sequence demonstrated a 90% identity on the nt as well as on the aa level with EqIFN- γ

2.3.2 Expression and purification of white rRhIFN- γ

The rRhIFN- γ protein with a hexa-histidine tag (his-6-tag) at its N-terminal end and the additional aa's derived from the GW recombination sequence and thrombin cleavage site was expressed in *E. coli* by plasmid pET15-RhIFN- γ . Upon IPTG induction a strong protein band with the expected molecular weight for the tagged rRhIFN- γ was induced (Fig. 2.3, Lane 3). The major part of the expressed rRhIFN- γ was present in the insoluble fraction as inclusion bodies (Fig. 2.3, Lane 5). After solubilization of the inclusion bodies in 8 M urea (Fig. 2.3, Lane 6), the majority of the his-6-tag rRhIFN- γ bound to a column with immobilized Ni²⁺ (some of the column matrix was removed and loaded, Fig. 2.3, Lane 8) and a minor part failed to bind and showed up in the flow-through fraction (Fig. 2.3, Lane 7). After washing, the bound protein was refolded on the column. After elution and dialysis the refolded IFN- γ had a purity of at least 95% (Fig. 2.3, Lane 9).

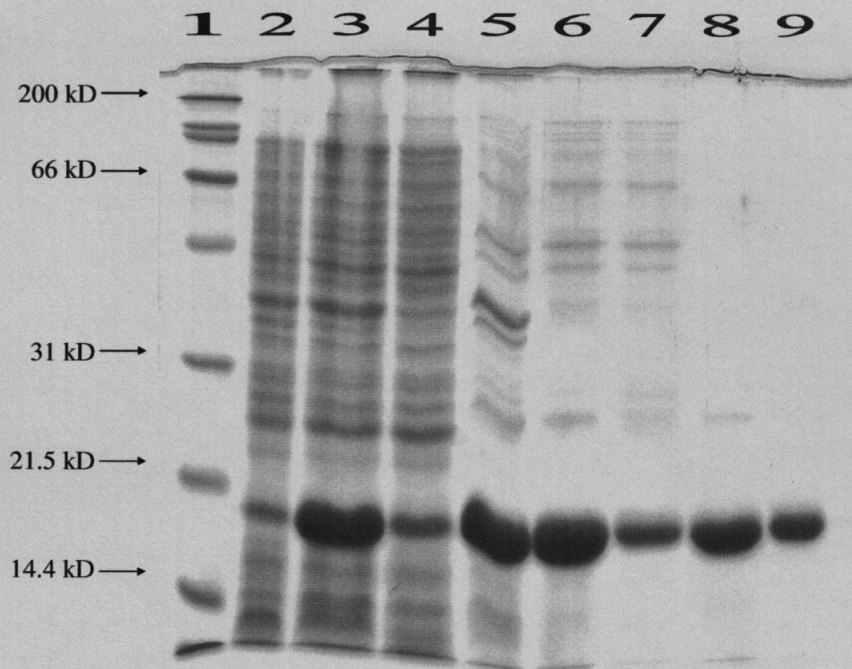


Figure 2.3 SDS-PAGE showing the purification of white rRhIFN- γ . **Lane 1:** Broad Range Mw Marker; **Lane 2:** Total bacterial lysate (uninduced); **Lane 3:** Total bacterial lysate (IPTG-induced); **Lane 4:** Soluble fraction; **Lane 5:** Insoluble fraction (inclusion bodies); **Lane 6:** Solved inclusion bodies (8 M urea); **Lane 7:** Flow-through Ni²⁺ column; **Lane 8:** Protein bound on column matrix; and **Lane 9:** Eluted protein

2.3.3 *Prototype capture ELISA for the detection of white RhIFN- γ*

The purified white rRhIFN- γ was used to generate specific monoclonal mouse antibodies and polyclonal chicken antibodies (IgY). The antibodies were used to develop a capture ELISA using monoclonal antibody 1H11 as a capture antibody and the anti-IFN- γ polyclonal IgY as detecting antibody. This capture ELISA was able to detect recombinant white RhIFN- γ (Fig. 2.4). The capture ELISA could also detect native IFN- γ as was demonstrated by the strong signal obtained with supernatants of PBMC of three white rhinoceroses that had been stimulated with Con A to induce the expression of this cytokine (Fig. 2.4).

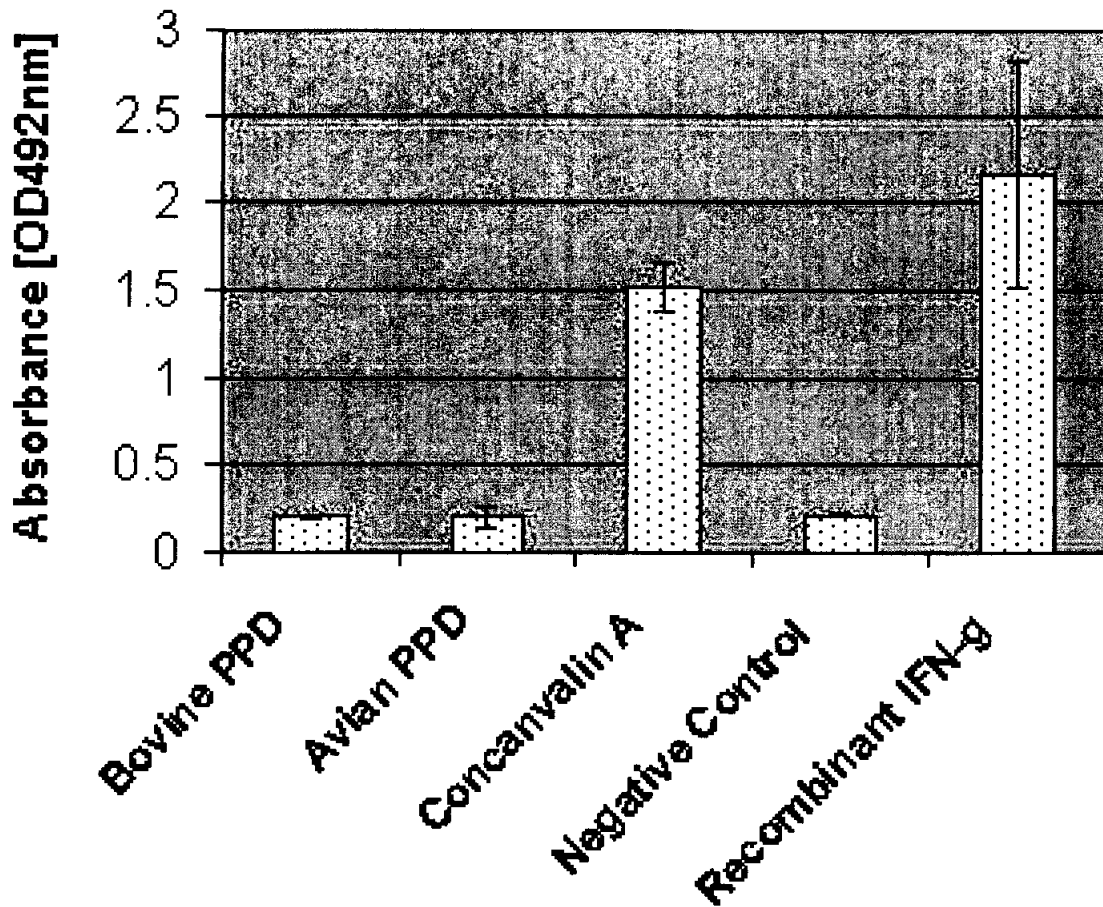


Figure 2.4 Detection of white RhIFN- γ in PBMC stimulated with Bovine PPD, Avian PPD and Con A using the IFN- γ capture ELISA. Results are expressed as the mean value from three rhinoceroses. "I" indicates the standard deviations

2.4 Discussion

Mycobacterium bovis has been found to have an exceptionally wide host range which includes domestic ruminants and captive and free-ranging wildlife (Buddle *et al.* 2000; Michel *et al.* 2006). The high BTB prevalence among buffalo herds in the southern region of the KNP has facilitated the spillover of *M. bovis* infections into a number of animal species and poses a real threat to valuable species, thus jeopardising breeding and relocation projects of, amongst others, rhinoceroses in the context of conservation and biodiversity. Although not yet diagnosed in South Africa, rhinoceroses have been reported to be susceptible to both *Mycobacterium tuberculosis* and *M. bovis* (Mann, Bush, Janssen *et al.* 1981; Dalovisio, Stetter & Mikota-Wells 1992; Stetter, Mikota, Gutter *et al.* 1995; Michel 2002), but little is

known concerning the pathogenesis of the disease in them. Diagnostic tests available for *M. bovis* infections are often limited to certain species or lack validation in others, such as the TST in pachyderms. As a consequence, the specificity and sensitivity of these tests are not known in these animal species. Control of infection in the individual animal or in groups of animals strongly depends on early diagnosis. The IFN- γ test has proven to be highly successful in demonstrating mycobacterial infections in domestic and non-domestic species, including cattle, goats, bison, African buffaloes (Bovigam™) (Grobler, Michel, De Klerk & Bengis 2002), deer (Cervigam™) (Waters, Palmer, Thacker *et al.* 2008) and primates (Primagam™) (Garcia, Yee, Bouley *et al.* 2004). In wildlife species the test is considered practical, as it requires minimal invasion and manipulation.

This chapter reports the successful cloning, sequencing and expression of IFN- γ from white rhinoceroses, and the production of monoclonal antibodies specific for rRhIFN- γ , as essential tools for development of assays to detect the IFN- γ response to *M. bovis* infection in this animal species. As expected from the close phylogenetic relationship, the highest homology was observed with the EqIFN- γ sequence on the DNA as well as the protein level (both 90%). The RhIFN- γ gene is predicted to encode a signal sequence of 25 aa's and a mature protein of 141 aa residues. For expression purposes in *E. coli*, the sequence encoding the mature protein was initially cloned. The forward primer that was used to clone the mature IFN- γ was based on the EqIFN- γ sequence, because of its relatedness to the rhinoceros. Potentially this equine forward primer might have had one or a few mismatches with the rhinoceros sequence, which would consequently result in different aa's at the N-terminal end of the mature white RhIFN- γ . The sequence of the 5' end was therefore cloned separately using forward primers corresponding to EqIFN- γ sequences upstream of the start codon and reverse primers based on the obtained white RhIFN- γ sequence. Based on the new set of primers derived from those sequences, the final nt at the 5' end was verified. Indeed, the equine forward primer used to clone the sequence encoding the mature RhIFN- γ contained two mismatches with the real rhinoceros sequence. Only the second mismatch results in a different amino acid after translation. This aa, the first of the predicted mature RhIFN- γ , turned out to be a valine (v) instead of the alanine (a) (Fig. 2.2). Apparently, the aa at this position does not form an essential part of the epitope recognized by monoclonal antibody 1H11, as a prototype capture ELISA could be developed, using this antibody in combination with polyclonal chicken antibodies, that is able to detect both recombinant and native white RhIFN- γ .

To date tests have only been performed in the white rhinoceros and have yet to be performed in black rhinoceroses (*Diceros bicornis*). A first preliminary validation of the specificity of the test was done on TB-free white rhinoceroses. No IFN- γ was detected after *M. bovis* stimulation; whereas it was shown that a signal was detected after Con A stimulation. Sensitivity of the tests is presently unknown and might be evaluated in *M. bovis*- / *M. tuberculosis*-infected rhinoceroses. In conclusion: although further development and validation of the described capture ELISA is necessary, it demonstrates a promising approach towards a diagnostic assay for *M. bovis* infection in rhinoceroses.

2.5 Publication

This chapter was published in the *Journal of Veterinary Immunology and Immunopathology* in 2007. Herewith is the reference:

Morar, D., Tijhaar, E., Negrea, A., Hendriks, J., van Haarlem, D., Godfroid, J., Michel, A.L. & Rutten, V.P.M.G. 2007. Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) interferon-gamma (IFN- γ) and the production of rhinoceros IFN- γ specific antibodies. *Journal of Veterinary Immunology and Immunopathology*, 115:146-154.

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