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THE EVALUATION OF A BCG VACCINE AGAINST BOVINE TUBERCULOSIS IN AFRICAN BUFFALOES (*SYNCERUS CAFFER*)

By

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degree MSc (Veterinary Science)

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DECLARATION

I, Lin-Mari de Klerk-Lorist, do hereby declare that apart from the assistance received, which has been duly acknowledged, this dissertation represents my own work and has not been submitted by me for another degree at any other University.

________________________ ________________________

CANDIDATE DATE

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DEDICATION

I dedicate this dissertation to my wonderful parents, Japie and Uysie de Klerk, for all their financial and emotional support during my career development. I thank them for their contribution to my academic achievements and for encouraging me to pursue my dreams.

I would also like to include my husband Rudi, and thank him for all his love and support during the field work – especially for tracking the buffalo escapees on the last day of the BCG Vaccine Study!

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ABBREVIATIONS

γ	Gamma
AFO	Acid-fast organism
APC	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
BTB	Bovine tuberculosis
cfu	Colony-forming units
cm	Centimeter
CMI	Cell-mediated immunity
CpG	Cytidine phosphate guanosine
DDA	Dimethyldeoctadecylammonium
DNA	Deoxyribonucleic Acid
DRL	Disease Research Laboratory
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked Immunosorbent Assay
EMEA	European Agency for the Evaluation of Medicinal Products
HD	High dose
IFN	Interferon
IL	Interleukin
IM	Intramuscular
INH	Isonicotinic hydrazide acid
IT	Intratracheal
IV	Intravenous
KNP	Kruger National Park
LD	Low dose
LJ	Löwenstein-Jensen
ln(n).	Lymph node(s)
LPS	Lipopolysaccharides

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MPL	Monophosphoryl Lipid A
nm	Nanometer
OD	Optical density
ODN	Oligodeoxynucleotides
OIE	Office International des Epizooties
OPD	Orthophenylenediamine dihydrochloride
OVI	Onderstepoort Veterinary Institute
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PI	Post infection
PPD	Purified protein derivative
RFLP	Restriction fragment length polymorphism
SC	Subcutaneous
T_H1	T-helper cell type 1
T_H2	T-helper cell type 2
TNF	Tumour necrosis factor
USDA	United States Department of Agriculture
ZN	Ziehl-Neelsen

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SUMMARY

THE EVALUATION OF A BCG VACCINE AGAINST BOVINE TUBERCULOSIS IN AFRICAN BUFFALOES (*SYNCERUS CAFFER*)

By

Lin-Mari de Klerk-Lorist

Supervisor: Prof. M. van Vuuren

Co-supervisor: Prof. N. P. J. Kriek

To assist in the evaluation of BCG vaccination in African buffaloes (*Syncerus caffer*), an infection model for *Mycobacterium bovis* was established, using an intratonsilar route of inoculation. Two groups of 11 buffaloes each, aged approximately 18 months, were infected with either 3,2 x 10^2 cfu (low dose) or 3 x 10^4 (high dose) of virulent buffalo strain *M. bovis*. A control group of six buffaloes received saline via the same route. The infection status was monitored using the intradermal tuberculin test, an ELISA and a modified interferon-gamma assay. All buffaloes were euthanased 22 weeks post infection and the development of lesions in the left retropharyngeal lymph node was evaluated by macroscopic examination, mycobacterial culture and histopathology. It was found that the high dose caused macroscopic lesions in 9 out of 11 buffaloes that were comparable to that observed in buffaloes with natural disease. *Mycobacterium bovis* was isolated from all animals in the high dose and from 6 out of 11 buffaloes in the low dose group.

The efficacy of a live BCG-Pasteur vaccine was tested in a group of buffalo calves captured in the northern districts of the Kruger National Park from herds with known negative tuberculosis status. Primary and booster vaccinations with BCG (1173P2) were administered to 15 calves, while another 15 were left unvaccinated as control animals. All the buffalo calves were challenged with the high dose of live *M. bovis* (as determined in the Infection Model) via intratonsilar inoculation. Laboratory tests were able to distinguish between infected and non-infected animals from an early stage. All buffaloes were euthanased 34 weeks after infection and the development of lesions in the lymph nodes of the head, thorax, carcass and abdomen was evaluated by macroscopic examination, mycobacterial culture and histopathology. The lungs were carefully palpated to detect the presence of tuberculous granulomas. Macroscopic lesions in the lymph nodes were found in 10 out of 14 control buffaloes and 7 out of 15 vaccinated animals. The lesions were comparable to that observed in buffaloes with natural infection. The lesion scores of individual animals were generally much higher in the BCG vaccine study than what was experienced with the Infection Model. *Mycobacterium bovis* was isolated from 12 out of 14 control animals and from 12 out of 15 vaccinated buffaloes. Although fewer vaccinated animals developed tuberculous lesions, the differences between the two groups were not statistically significant and it can be concluded that under the prevailing conditions the BCG vaccine was unable to protect buffalo calves against the establishment of *M. bovis* infection.

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OPSOMMING

DIE EVALUERING VAN 'N BCG ENTSTOF TEEN BEES TUBERKULOSE IN DIE AFRIKA BUFFEL (*SYNCERUS CAFFER*)

Deur

Lin-Mari de Klerk-Lorist

Promotor: Prof. M. van Vuuren

Mede-promotor: Prof. N. P. J. Kriek

'n Infeksie model vir bees tuberkulose in die Afrika buffel (*Syncerus caffer*) is ontwikkel deur lewendige *Mycobacterium bovis* organismes in die mangelholte in te spuit. Twee groepe van 11 buffels elk, met 'n gemiddelde ouderdom van ongeveer 18 maande, is besmet met óf die lae dosis (3,2 x 10^2 kve) óf die hoë dosis (3 x 10^4 kve) van 'n virulente buffel stam *M. bovis*. 'n Kontrole groep van ses buffels het 'n soutoplossing via dieselfde roete ontvang. Die intradermale tuberkulien toets, 'n ELISA en 'n aangepaste gamma interferon toets wat reeds voorheen beskryf is, is gebruik om die infeksie status van die diere te bepaal. Al die buffels is 22 weke na die besmetting van kant gemaak, en die ontwikkeling van letsels in die linker retrofaringeale limfknoop is volgens makroskopiese ondersoek, mikobakteriële kweking en histopatologie beoordeel. Die hoë dosis het makroskopiese letsels in 9 van die 11 buffels veroorsaak. Hierdie letsels het baie goed vergelyk met tering letsels wat gewoonlik aangetref word in buffels wat natuurlik besmet is. Lewendige *M. bovis* is geïsoleer vanuit al die diere in die hoë dosis en uit 6 van die 11 buffels in die lae dosis groep.

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Die effektiwiteit van ‘n lewendige BCG-Pasteur entstof is in ‘n groep buffel kalwers getoets wat in die noordelike distrikte van die Kruger Nasionale Park gevang is. Hierdie diere is spesifiek geselekteer uit kuddes bekend vir hulle negatiewe tuberkulose status. Primêre en sekondêre entings met BCG (1173P2) is aan 15 kalwers toegedien, terwyl ‘n verdere 15 diere nie geënt is nie, om as kontrole diere te dien. Die hoë dosis (soos aangedui deur die Infeksie Model) is gebruik om die buffel kalwers te besmet. Laboratorium toetse kon reeds vanaf ‘n vroëe stadium tussen siek en gesonde diere onderskei. Al die buffels is 34 weke na besmetting van kant gemaak en die letsels wat in die limfknope van die kop, bors, karkas en buik ontwikkel het, is deur middel van makroskopiese ondersoek, mikobakteriële kweking en histopatologie beoordeel. Die longe is deeglik betas om vas te stel of enige tuberkulose granulome teenwoordig was. In die kontrole groep het 10 van die 14 diere makroskopiese letsels in die limfknope gehad, terwyl 7 van die 15 diere wat geënt is TB letsels getoon het. Alhoewel die letsels vergelykbaar was met die patologie van buffels wat natuurlik besmet is, was die letsel tellings van die individuele diere heelwat hoër in die BCG entstof studie as wat in die Infeksie Model ondervind is. Daar is positiewe *M. bovis* isolasies gemaak vanuit 12 van die 14 kontrole diere sowel as 12 van die 15 buffels wat geënt is. Alhoewel daar minder makroskopiese TB letsels was in die geënte buffels, was die resultate nie statisties betekenisvol nie. Dus kan afgelei word dat die BCG enstof onder die teenwoordige omstandighede nie in staat was om die buffels teen *M. bovis* besmetting en die daaropvolgende letsel ontwikkeling te beskerm nie.

CHAPTER I

1 INTRODUCTION

Bovine tuberculosis (BTB) caused by *Mycobacterium bovis*, is an exotic disease that is spreading amongst the buffalo (*Syncerus caffer*) herds in the Kruger National Park (KNP). It was first diagnosed during July 1990 in a young buffalo bull that was found near the Maqili picket in the Stolznek region, close to the southwestern boundary (Bengis, Kriek, Keet, Raath, de Vos & Huchzermeyer 1996). Although annual reports of the State Veterinarian from the Barberton district suggested that the disease entered the KNP across the Crocodile River (southern boundary) during the early 1960s as a result of contact between infected cattle and buffaloes, the exact manner of entrance is still not clear. It is assumed that from the Crocodile Bridge area BTB gradually spread throughout the south of the park as well as into the central districts. BTB is estimated to be spreading north through the KNP at a rate of 6km/year (De Vos, Bengis, Kriek, Michel, Keet, Raath & Huchzermeyer 2001).

The disease probably spread further than was reported by veterinarians and field rangers during the late nineties. Grobler, Michel, De Klerk & Bengis (2002) reported that buffalo herds north of Letaba rest camp were already infected with BTB by the year 2000. Buffaloes as far north as Shingwedzi and a lioness at Mingerhoutdam have tested positive for BTB (Grobler *et al* 2002; Keet 2001, unpublished). Spread of the disease to different areas was most likely made possible because of buffalo behaviour previously recorded in the KNP. This mainly includes the fact that buffalo herds integrate with one another, where herds break up into smaller groups and then again aggregate at large waterholes (Bengis *et al* 1996). The presence of the disease may in time influence the ecology of endangered species in the park (De Vos *et al* 2001).

Buffaloes have now become the main reservoir hosts of BTB in the KNP. The disease is of great concern because of its exotic nature and almost unlimited host

range (Bengis 1996). The number of BTB-positive spill-over species in the KNP has increased and now includes lion (*Panthera leo*), greater kudu (*Tragelaphus strepsiceros*), baboon (*Papio ursinus*), leopard (*Panthera pardus*), cheetah (*Acinonyx jubatus*), spotted hyena (*Crocuta crocuta*) and honey badger (*Mellivora capensis*). In private reserves neighbouring the KNP species such as the warthog (*Phacochoerus aethiopicus*), bush pig (*Potamochoerus porcus*) and genet (G*enetta genetta*) were also reported to be infected (Keet, Kriek, Bengis, Grobler & Michel 2000; Keet, Kriek, Bengis & Michel 2001). The epidemiology of the disease in the KNP has been discussed by De Vos *et al* (2001), while Bengis (1996) stated that social, gregarious species, predators and scavengers would be at greatest risk of becoming infected with BTB.

Eradication of BTB from wild animal populations is very difficult. The question is often asked whether treatment of infected animals would be possible to prevent the spread of the disease. Although isonicotinic acid hydrazide (INH) is an aid in the treatment of tuberculosis in cattle, it must be administered daily for at least 2-3 months. A 78% bacteriological cure may be theoretically possible, but not all treated animals are cured, and INH cannot be used as a means of eradicating the disease (Kleeberg 1967). Control and eradication of BTB within the KNP is difficult if not impossible, due to the extensive geographical ranges that the different infected buffalo herds occupy. The treatment of individuals in an infected free-ranging buffalo herd is not possible. It has been suggested that BTB in the KNP may be controlled by concurrent vaccination of buffalo calves under the age of two years to protect them against BTB, with culling of severely diseased animals to lessen the environmental contamination.

The efficacy of the bacillus Calmette-Guérin (BCG) vaccine has been tested in a number of species (Buddle, Skinner & Chambers 2000; Corner, Buddle, Pheiffer & Morris 2001; Corner, Buddle, Pheiffer & Morris 2002). Griffin, Chinn, Rodgers & Mackintosh (2001) reported a high success rate using a BCG vaccine in red deer (*Cervus elaphus*) provided that a booster was given eight weeks after primary

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vaccination. A single vaccination resulted in fewer diseased animals as well as fewer macroscopic lesions observed at necropsy, while a booster vaccination prevented the establishment of infection. Although the BCG vaccine did not give complete protection against tuberculosis it decreased the severity of the disease and lowered mycobacterial contamination of the environment (Cross, Labes, Griffin & Mackintosh 2000). As a result other animals are less likely to become infected. Vaccination can thus be usefully incorporated into the control of TB wherever animals of high economic, social or conservation value are involved and culling programmes are not appropriate (Suazo, Escalera & Gallegos Torres 2003).

No new candidate vaccine has been able to improve on the ability of the BCG vaccine to stimulate a protective immune response against bovine tuberculosis (Suazo *et al* 2003), and therefore the BCG 1173P2 vaccine was selected for the purpose of this study. However, the efficacy of any vaccine can only be determined by means of a proper challenge trial following vaccination. An experimental infection model that reproduced the disease with the typical range of lesions has been described in red deer (Mackintosh, Waldrup, Labes, Buchan & Griffin 1995; Griffin, Mackintosh & Buchan 1995) and in domestic cattle (Palmer, Whipple, Rhyan, Bolin & Saari 1999). These challenge models mimicked the natural lesions of the disease seen in animals infected with bovine tuberculosis (Griffin *et al* 1995).

This study was based on findings from previous vaccine studies done on several different mammalian species in New Zealand, and the objectives included:

1) To develop an infection model that would simulate natural BTB infection with typical tuberculous lesion development in African buffaloes.

2) To evaluate the efficacy of the BCG vaccine against BTB in buffalo calves.

CHAPTER II

2 LITERATURE REVIEW

2.1 Bovine tuberculosis in wildlife

Bovine tuberculosis has been reported in captive and free-living wild animals worldwide (Huchzermeyer, Brückner, van Heerden, Kleeberg, van Rensburg, Koen & Loveday 1994; Thoen, Schliesser & Körmendy 1995). The host range among non-domestic animals appears to be unlimited (Francis 1958). Bovine tuberculosis has been diagnosed in New Zealand in red deer (*Cervus elaphus*), fallow deer (*Dama dama*), possums (*Trichosurus vulpecula*) and ferrets (*Mustela furo*) all of which could play a role in the transmission of BTB to domestic stock (Coleman, Jackson, Cooke & Grueber 1994). *Mycobacterium bovis* infection is also present in fallow deer in Sweden. Badgers (*Meles meles*) are considered to be the maintenance hosts of BTB in England and Ireland (Gallagher, Muirhead & Burn 1976; Hewson & Simpson 1987; Cheeseman, Wilesmith & Stuart 1989; Hardie & Watson 1992), while red deer have also been diagnosed with BTB in England and Hungary. Tuberculosis has been described in farmed water buffaloes (*Bubalus bubalis*) and camels (*Camelus dromedarius*) in Egypt, while farmed elk (*Alces alces*) and free-ranging bison (*Bison bison*) in Canada and the USA are seen as additional maintenance hosts (Fanning & Edwards 1991; Tessaro, Forbes & Turcotte 1990). *Mycobacterium bovis* is maintained within populations of free-ranging cervids like sika deer (*Cervus nippon*), white-tailed deer (*Odocoileus virginianus*) and fallow deer in North America without the involvement of domestic livestock (Schmitt, Fitzgerald, Cooley, Burning-Fann, Sullivan, Berry, Carlson Minnis, Payeur & Sikarskie 1997).

Bovine tuberculosis has been diagnosed in several wild animal species in southern Africa (De Vos *et al* 2001). In the eastern Cape Province of South Africa, Paine & Martinaglia (1928) reported the first cases of tuberculosis in greater kudu

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(*Tragelaphus strepsiceros*) and the common duiker (*Sylvicapra grimmia*), caused by infection with *Mycobacterium bovis*. Thorburn & Thomas (1940) also reported on the presence of the disease in the Eastern Cape greater kudu population. A case of mycobacteriosis in a free-ranging black rhinoceros (*Diceros bicornis*) in the Hluhluwe Game Reserve in Kwazulu-Natal was reported by Keep & Basson (1973). More recently, BTB was reported in buffaloes and kudus in the Hluhluwe/Umfolozi Park in Kwazulu-Natal (Flamand 1993; Cooper 1998). Further afield in Africa, BTB was diagnosed in free-ranging lechwe (*Kobus leche kafuensis*) in Zambia (Gallagher, MacAdam, Sawyer & Van Lavieren 1972; Clancey 1977). Guilbride, Rollinson, McAnulty, Alley & Wells (1963) reported the first case of BTB in free-ranging buffaloes in Uganda. Woodford (1972) surveyed the Queen Elizabeth National Park and confirmed the presence of BTB in the buffalo population there. He later reported that the disease had become endemic in the buffaloes with spill-over into the warthog population. A small percentage of mortalities in buffaloes were recorded every year (Woodford 1982).

2.2 Bovine tuberculosis in the Kruger National Park

During the early 1990s BTB was diagnosed in African buffaloes (*Syncerus caffer*) in the southern districts of the Kruger National Park. The index case was reported to the state veterinarian by the local ranger. The 2-year-old buffalo bull was emaciated and moribund and therefore euthanased. The necropsy revealed a severe granulomatous inflammatory reaction in the lungs, as well as enlargement of the pulmonary lymph nodes due to a similar granulomatous reaction and histological lesions typical of a mycobacterial infection (Bengis *et al* 1996). A follow-up survey involving necropsies in buffalo herds in the surrounding areas, revealed widespread BTB in the southern herds in the KNP (Bengis & Keet 2000). Guilbride *et al* (1963) and also Woodford (1982) ascribed the success of transmission of BTB to the gregarious nature of buffaloes. They are now believed to be the maintenance host of BTB in the KNP (De Vos *et al* 2001).

Although BTB can potentially infect any mammalian species in the KNP, the social and gregarious species, as well as predators and scavengers would predictively be at greatest risk (Bengis 1996). Spill-over of the disease to a number of other wildlife species including the cheetah (*Acinonyx jubatus*), lion (*Panthera leo*), baboon (*Papio ursinus*), kudu (*Tragelaphus strepsiceros*), leopard (*Panthera pardus*), warthog (*Phacochoerus aethiopicus*), badger (*Mellivora capensis*) and hyena (*Crocuta crocuta*) has been reported (Keet, Kriek, Penrith, Michel & Huchzermeyer 1996; Bengis & Keet 2000; Keet, Kriek, Bengis & Michel 2001). *Mycobacterium bovis* was also isolated from one large spotted genet (*Genetta trigina*) as well as a bushpig (*Potamochoerus porcus*) in a conservation area neighbouring the KNP (Bengis & Keet, State Veterinary Department Skukuza, unpublished data).

2.3 Treatment and control in domestic animals and wildlife

Several countries have implemented large-scale campaigns to eradicate bovine tuberculosis. The two most widely used methods of control are the test-and-removal method, and the Bang method, where diseased animals are kept isolated and gradually eliminated from the infected herd to create a “clean” herd (Meyers & Steele 1969; Huchzermeyer *et al* 1994). The test-and-slaughter policy is the most successful approach in controlling and eradicating this disease (Huchzermeyer *et al* 1994; Bourne, Donnelly, Cox, Gettinby, McInerney, Morrison & Woodroffe 2000; Perez, Ward & Ritacco 2002). During the late 1970s there was a high prevalence of tuberculosis in the feral pig population of the northern territory of Australia due to close contact between pigs and cattle. Selective or total depopulation was used in cattle as well as water buffalo herds in Australia and the status of a 'TB Free Area' was achieved in December 1997 (Cousins & Roberts 2001). After the implementation of this tuberculosis eradication scheme, follow-up surveys indicated that the prevalence of disease in feral pigs declined to less than 1%. This also confirmed the belief that the feral pigs were dead-end hosts and if the disease was controlled in the reservoir animals it would eventually also disappear from the spill over species (McInerney, Small & Caley 1995).

Although complete depopulation was the preferred control measure of TB-infected herds in the United States, a decision was taken by producers and regulatory officials to use test-and-removal rather than complete depopulation (Schoenbaum & Meyer 1995; Collins 1996). Perez *et al* (2002) reported that whenever the disease prevalence was higher than 22%, all control strategies were less efficient than herd depopulation. Attempts to eradicate tuberculosis from cattle and farmed deer in some countries have been frustrated by the existence of wildlife reservoirs of *Mycobacterium bovis* (Buddle, Skinner & Chambers 2000b).

In certain deserving cases where pedigree animals or rare species in zoological collections are infected, permission may be granted for treatment of the infected animals. Isonicotinic acid hydrazide (INH) can be used for treatment but does not represent a means of eradicating the disease because not all reactors are cured of the mycobacterial infection (Huchzermeyer *et al* 1994; Thoen, Schliesser & Körmendy 1995). The effectiveness of INH treatment in cattle is superior to that of any other drug if administered at 10mg/kg body weight daily for eight weeks. Intermittent medication is less effective than daily dosing (Kleeberg 1967). Isonicotinic acid hydrazide is an inexpensive drug with minimal side effects and can therefore be used with relative safety. Generally a higher dosage rate is used in monkeys and great apes than when hoofed animals such as kudus, oryxes and llamas are treated (Ward, Elwell, Tingpalapong & Pomsdhit 1985).

The discovery of streptomycin in 1944 was a major breakthrough in TB drug development where *M. tuberculosis* was concerned. During the course of the next three decades all the other drugs such as cycloserine, kanamycin, rifampicin, ethambutol and pyrazinamide used in TB treatment were also introduced (Duncan 2003). Consideration should be given to using multiple anti-tuberculosis drugs when treating non-responsive animals with signs of progressive disease (Wolf, Gibson, Watson & Baskin 1988; Haagsma 1992). A combination of INH and streptomycin was successful in controlling clinical disease in monkeys (Ward *et al* 1985), while anti-tuberculosis drugs for humans have also been used for treating

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disease in monkeys, great apes and other animals in captivity (Wolf *et al* 1988). Ethambutol has been used to treat *M. bovis* infections in White Arabian Oryxes, while rifampicin was successfully used in mink (Haagsma 1992).

Most wild species are difficult to restrain and more information is needed on the route of administration and dosage of anti-tuberculosis drugs necessary to maintain adequate blood levels to destroy tubercle bacilli in them. The importance of TB treatment in captive wild animals is emphasized by the difficulty of replacing endangered species, as well as the desire to preserve valuable blood lines (Thoen *et al* 1995). Treatment strategies are very intensive and in the majority of species a treatment period of one year is recommended (Ward *et al* 1985). Such an intensive treatment programme would be impossible to implement in an area where the animals are ranging free.

2.4 Laboratory diagnostics

Intradermal tuberculin testing is a key factor in most field studies, but blood assays may offer opportunities in overcoming the limitations of skin testing caused by animal desensitization and anergy in the later stages of infection. Maximum disease detection could possibly be achieved by appropriate use of available blood assays in conjunction with skin testing (Neill, Hanna, Clements, Cassidy, Pollock & Bryson 1995; Menzies & Neill 2000). Sensitivity and specificity of 85 and 93 percent, respectively, was measured in the IFN-γ assay used in cattle herds in New Zealand. It was also shown that the IFN-γ test, unlike skin testing could accurately predict the animal's *M. bovis* infection status at short intervals following skin testing (Rothel, Corner, Cox & Wood 1992; Doherty, Monaghan, Bassett & Quinn 1995). Ryan, Buddle & De Lisle (2000) showed that high levels of sensitivity and specificity could be achieved with the IFN-γ test when examining samples only 8 to 28 days after intradermal tuberculin testing.

In free-ranging buffaloes the comparative intradermal tuberculin test is expensive and cause handling stress due to repeat immobilization and containment in a

boma. These animals often refuse to drink after capture, and become dehydrated. It appears that the state of dehydration compromises the interpretation of the skin test (Raath, Bengis, Bush, Huchzermeyer, Keet, Kernes, Kriek & Michel 1993). The IFN-γ test has been used and evaluated in buffaloes in South Africa to overcome problems associated with the skin test and to avoid culling of healthy buffaloes (Grobler *et al* 2002). Raath *et al* (1993) reported that the IFN-γ test had similar sensitivity and specificity ranges to the comparative intradermal tuberculin test. During the 2000 BTB survey the diagnosis of bovine tuberculosis in buffalo herds was, for the first time, based exclusively on the IFN-γ test (Grobler *et al* 2002). The strong correlation between test-positive and culture-positive buffaloes confirms the high specificity of the IFN-γ test (99.3%) found in the comparative field evaluation (Michel, Nel, Cooper & Morobane 2000).

It has been shown that lymphocyte transformation (LT) reactions can be used to diagnose TB in monkeys (Chaparas, Hedrick, Clark & Garman 1970), cattle (Muscoplat, Thoen, Chen & Jonsone 1975) and camelids (Thoen, Temple & Johnson 1988). The Disease Research Laboratory at the University of Otago (Dunedin, New Zealand) determined the level of sensitivity for the LT at 90.7% when applied in red deer (*Cervus elaphus*). A combination of the ELISA and LT assay is now used in New Zealand to determine the TB status of an individual animal (Griffin, Cross, Chinn, Rodgers & Buchan 1994). The ELISA is specific for the detection of circulating antibodies against bovine purified protein derivative (PPD). The combination of these tests incorporates the measurement of the relative humoral and cellular immunological responses to *M. bovis* and *M. avium* antigens to provide a composite test with high levels of sensitivity (95%) and specificity (98%). The performance of the ELISA, however, is dramatically influenced by the intradermal tuberculin test, in that a much higher sensitivity is found with the ELISA 10 days after the tuberculin test (85.3%) than before (45.7%) the test (Griffin *et al* 1994).

2.5 Immune responses associated with mycobacterial infections

Studies in the 1940s in laboratory animals demonstrated that immunity to tuberculosis is mediated by cellular rather than humoral responses (Andersen 1997). The elimination of *M. bovis* from the tissues of a host depends, in part, on killing the bacillus in mononuclear macrophages that have been activated by soluble substances produced and released by T-lymphocytes (Thoen & Bloom 1995). Macrophage inflammatory and immune responses in red deer are similar to those in other mammalian species and macrophages play an important role in resistance to mycobacterial infections (Cross, Thomson, Slobbe, Griffin & Buchan 1996). Deficiencies of T-lymphocyte function and mononuclear dysfunction have been associated with opportunistic mycobacterial diseases (Mason, Greenberg, Yeu & Kirkpatrick 1982; Dannenberg 1989). Therefore, Orme & Collins (1983) considered T cell-mediated immunity essential for clearance of mycobacterial infections.

The roles of T cell subpopulations in BTB infection have included monitoring changes in peripheral blood lymphocytes following experimental infection with live *M. bovis* (Pollock, Pollock, Campbell, Girvin, Crockard, Neill & Mackie 1996; Cassidy, Bryson, Pollock, Evans, Forster & Neill 1998). In order to understand the protective role of T cells, it is essential to define not just the distribution of T cell subpopulations but also their functional capabilities (Liébana, Girvin, Welsh, Neill & Pollock 1999). The key cellular responses that determine the outcome of infection with virulent mycobacteria are related to the nature of the cytokines produced locally by the immune and other cell types.

T-helper cells can be divided into two distinct populations based on cytokine profiles that determine their functional role (Romagnani 1992). T-helper type 2 cells (T_H2) produce interleukin-4 (IL-4), necessary to regulate antibody production while T-helper type 1 cells (T_H1) produce IFN-γ and IL-2 and are associated with cellular resistance. Interferon-gamma (IFN-γ) is recognised to have a key role in anti-mycobacterial immunity, including activation of the microbicidal mechanisms of

macrophages. Investigations into the sources of IFN-γ in bovine tuberculosis have shown that both $CD4^+$ and $CD8^+$ T cells are significant producers of this cytokine (Liébana *et al* 1999). It has been shown that antigen-stimulated lymphocytes are capable of releasing metabolically active *M. bovis* bacilli from infected macrophages, thus providing strong evidence that cytolytic activity is a feature of cell-mediated immune (CMI) responses in bovine tuberculosis (Liébana, Aranaz, Aldwell, McNair, Neill & Pollock 2000). Other populations of cells are also considered to be involved in anti-mycobacterial immunity but the relative importance of each type of cell is not well understood (Buddle, Pollock, Skinner & Wedlock 2003).

T_H1 and T_H2 cells operate in a reciprocal fashion, whereby cellular and humoral immune responses are mutually antagonistic. T_H2 cells are thus associated with exacerbation and rapid lesion formation in several models of chronic infectious disease (Thoen & Bloom 1995). Griffin, Mackintosh and Buchan (1995) also indicated that IL-4 expression was only found in diseased animals or deer vaccinated with killed BCG in oil adjuvant, suggesting that IL-4, similar to specific antibody, is a sensitive diagnostic indicator of tuberculosis in deer and is compatible with the activation of T_H2 cells. Although humoral antibodies are not considered of importance in protecting the host from virulent tubercle bacilli, certain monoclonal antibodies prolonged survival in *M. bovis* infected mice and were also associated with reduction in granuloma size (Glatman-Freedman 2003). The existence or absence of an immune response does not predict resistance, but is dependent upon the balance between the various types of immune responses of the host and the virulence of the bacterial strain for that host (Thoen & Bloom 1995). It is therefore generally accepted that an understanding of anti-mycobacterial T-cell responses is of great importance in the rational development of vaccines for bovine tuberculosis (Buddle *et al* 2003).

There has been increasing interest in the relationship between innate and acquired immune responses to tuberculosis and the influence of this interaction on the

development of protective immunity (Orme 2001). Although this may have a fundamental effect on vaccine efficacy, its role has not often been considered for bovine tuberculosis. There is also increasing evidence that the anti-mycobacterial functions of individual populations of cells cannot be replaced completely by another population (Tascon, Stavropoulos, Lukacs & Colston 1998). Although a precise definition of the components of the overall immune response required for protection against bovine tuberculosis is still lacking, this concept has major implications, not only for the selection of vaccine candidates, but also for any adjuvant system by which they are made available to the immune system (Elhay & Andersen 1997).

2.6 Vaccination of wildlife

Although the ultimate goal is to eradicate BTB in wildlife reservoirs, the utilization of an effective tuberculosis vaccine is a promising option for the control of *M. bovis* infection in wildlife reservoirs (Buddle, Skinner & Chambers 2000a). In controlling the disease, the immediate aim is to reduce the spread of infection among the reservoir hosts and to other species. The spread of infection from diseased to uninfected animals can only be interrupted if the excretion of bacilli is reduced or prevented. To achieve this, the vaccine does not have to prevent primary infection, but it should rather reduce the shedding of mycobacteria to below the level required to sustain BTB infection in these populations (Buddle *et al* 2000a). The need for an effective TB vaccine for animals is great, but the target species for such a vaccination programme will probably vary from one country to another (McMurray 2001).

The efficacy of a vaccine for wildlife should preferably be evaluated in the target species itself (Buddle *et al* 2000a). Due to the low levels of natural transmission of tuberculosis under field conditions, experimental infection should be used to mimic the natural disease. This approach has the additional advantage of excluding complications associated with prior tuberculosis infection, an intangible factor that may confound field studies of vaccine efficacy (Griffin 2000). The intra-tonsilar

method has been shown to be effective in creating BTB in cattle (Palmer *et al* 1999) and red deer (Mackintosh *et al* 1995; Griffin *et al* 1995).

At present, research is being done to provide useful information on the practical issues that are central to the successful vaccination of wildlife (Griffin *et al* 1995; Buddle *et al* 2000). A vaccine should provide long-term protective immunity, ideally after a single immunization. It should also be cost-effective and have an appropriate delivery system. The vaccination of rare and endangered species in the Kruger National Park against anthrax and the programme against rabies throughout the world suggest that the concept is feasible.

2.7 Different approaches to vaccination

Vaccines against bovine tuberculosis can be divided into four major categories, namely 1) vaccines based on live, attenuated micro-organisms, 2) killed micro-organisms, 3) subunit vaccines composed of purified mycobacterial proteins, peptides or polysaccharide antigens, and 4) DNA vaccines (Buddle *et al* 2003). Much of the literature about the vaccination of animals against TB is focused on optimizing the route, dose and method of delivery of conventional BCG vaccines when administered to cattle, deer and other wildlife species (Aldwell, Keen, Stent, Thomson, Yates, de Lisle & Buddle 1995; Buddle, Keen, Thomson, Jowett, McCarthy, Heslop & de Lisle 1995; Cross, Labes, Griffin & Mackintosh 2000), but there have also been efforts to develop vaccines from attenuated mutants of *M. bovis* (Wedlock, Aldwell, Collins, de Lisle, Wilson & Buddle 1999). There is ongoing development in the subunit and DNA vaccine groups, and these new generation TB vaccines are currently being tested in mice and guinea pigs (Orme 1999).

2.7.1 DNA vaccines

Mycobacterial DNA vaccines have shown some promise in inducing protection against tuberculosis in mice by stimulating IFN-γ, cytotoxic T cell, and memory responses (Tascon, Colston, Ragno, Stavropoulos, Gregory & Lowrie 1996;

Lowrie, Silva, & Tascon 1997; Baldwin, D'Souza, Roberts, Kelly, Frank, Lui, Ulmer, Huygen, McMurray & Orme 1998). One of the approaches in the use of DNA vaccines, is to use genes that encode mycobacterial proteins and are incorporated into plasmids with mammalian promoters, and then injected into the skin or muscle of the experimental animals. It was reported that when such a DNA vaccine was given to mice, the vaccinated animals were more resistant to aerosol challenge than the control animals (Huygen, Content, Denis, Montgomery, Yawman, Deck, Dewii, Orme, Baldwin, D'Souza, Drowart, Lozes, Vandenbussche, Van Vooren, Liu & Ulmer 1996). DNA vaccines also have the advantage of being relatively safe and not requiring an adjuvant (Buddle *et al* 2003). Long-lived humoral and cellular immune responses were induced in mice, with this type of vaccine (Donnelly, Ulmer, Shiver & Liu 1997).

When tested in cattle, DNA vaccines expressing the mycobacterial antigens MPB70 and MPB83, induced $CD4^{+}$ T-cell responses, IgG1-biased humoral responses but only weak IFN-γ responses to bovine PPD. Calves vaccinated with the MPB83 expressing-plasmid demonstrated stronger cellular responses than those vaccinated with MBP70. Another important observation made, was that the specificity of the tuberculin skin test was not compromised by DNA vaccination (Vordermeier, Cockle, Whelan, Rhodes, Chambers, Clifford, Huygen, Tascon, Lowrie, Colston & Hewinson 2001). However, when the experimental animals were challenged with live *M. bovis* during a challenge experiment, these vaccines did not induce protection. Even when boosting the DNA-vaccinated animals with MPB70 protein, only humoral responses were enhanced, which did not result in protection against infection (Vordermeier *et al* 2001; Buddle *et al* 2003).

Tascon *et al* (1998) reported that a plasmid DNA vaccine encoding mycobacterial HSP65 antigen protected mice against *Mycobacterium tuberculosis* challenge. This vaccine, when given therapeutically, reduced bacterial loads in mice infected with *M. tuberculosis*. Recently, prime-boost strategies have been developed to improve the efficacy of DNA vaccines and are based on the use of DNA vaccines to prime

the immune system, which is then boosted with proteins, peptides or live attenuated vaccines (Degano, Schneider, Hannan, Gilber & Hill 1999; McShane, Brookes, Gilbert & Hill 2001; Tanghe 2001). Protective cell-mediated immune responses as well as protection against tuberculosis in small animal models were demonstrated when a live booster vaccine expressing similar antigens to the primary agent was used (Feng, Palendira, Demangel, Spratt, Malin & Britton 2001; McShane *et al* 2001).

A strategy where DNA vaccines were used as priming agents, followed by BCG boosting resulted in a significant reduction of the number of diseased animals. In contrast, the BCG vaccine alone induced less protection while the DNA vaccine alone induced no protection at all (Buddle *et al* 2003). Vordermeier, Lowrie & Hewinson (2003) demonstrated that strong, more homogenous, cellular immune responses were induced in cattle primed with a plasmid DNA vaccine and boosted with a recombinant protein in adjuvant. DNA prime-protein boost vaccination also resulted in stronger humoral responses with a more balanced IgG profile compared to any of the vaccines being used alone. However, the above-mentioned vaccine strategy has not been evaluated in experiments where cattle were challenged with live *M. bovis* (Vordermeier *et al* 2003). Another interesting observation made by Vordermeier *et al* (2001), was that DNA vaccines administered via the intramuscular route stimulated stronger cellular responses than intradermal immunization.

2.7.2 Mycobacterial protein vaccines

The vaccination of mice and guinea pigs with culture filtrate proteins of *M. bovis* and *M. tuberculosis* demonstrated that these vaccines can induce protection against aerogenic challenge with *M. tuberculosis* and *M. bovis* (Andersen 1994; Baldwin *et al* 1998; Bosio & Orme 1998). The fact that protein vaccination may not induce reactivity to the tuberculin skin test makes the concept of using these vaccines attractive. The effectiveness of the tuberculosis protein vaccines may also not be influenced by prior environmental mycobacterial sensitization (Brandt,

Cunha, Olsen, Chilima, Hirsch, Appelberg & Andersen 2002). For protection against mycobacterial diseases in cattle, one needs to induce strong cellular immune responses and this is very difficult to achieve when using protein vaccines. Andersen (1994) reported that dimethyldeoctadecylammonium chloride (DDA) served as an effective adjuvant for mycobacterial protein vaccines in mice, but it was unable to induce cellular immune responses in cattle (Wedlock, Keen, McCarthy, Andersen & Buddle 2002).

Another important approach is the use of various proteins released into the filtrate as subunit vaccines in combination with adjuvants, which are added to drive the required T_H1 response. No effective adjuvants or antigen delivery systems for inducing effective T_H1 responses in humans against *M. tuberculosis* have been found. These formulations have been shown to be protective, but both protection and long-term survival have been less than what were seen when using BCG (Hubbard, Flory & Collins 1992; Andersen 1994).

However, this situation may change as protein-based vaccines are entering clinical trials (Reed, Alderson, Dalemans, Lobet & Skeiky 2003). There is considerable interest in the identification of adjuvants and immunostimulants that assist in inducing CMI responses in cattle. These findings may also have particular relevance for the control of diseases caused by other intracellular pathogens (Buddle *et al* 2003). Adjuvants currently used with licensed vaccines are not effective T-cell adjuvants, and therefore do not have potential for use in TB vaccines. Some preparations, such as Freund's incomplete adjuvant, are more effective in inducing CMI responses, but are often toxic (Buddle *et al* 2003). Without safe and effective T-cell adjuvants, a new generation of vaccines and immunotherapeutics for diseases such as malaria and tuberculosis will not be possible (Reed *et al* 2003).

Corixa's Monophosphoryl Lipid A (MPL) is a chemically modified and biologically attenuated derivative of lipopolysaccharide (LPS) from *Salmonella minnesota.* It

stimulates and activates antigen-presenting cells (APCs), leading to the production of cytokines including TNF-α, IFN-γ and IL-12. These cytokines have all been implicated in protective immune responses against *M. tuberculosis*. The most effective adjuvants in inducing protective immune responses with protein-based subunit vaccines against TB in animal models contain MPL (Reed *et al* 2003). The incorporation of bovine recombinant IL-2 in an *M. bovis* culture filtrate protein vaccine with lipid A adjuvant markedly enhanced antigen-specific antibody responses and induced weak antigen-specific IFN-γ responses in cattle (Wedlock, Vesosky, Skinner, De Lisle, Orme & Buddle 2000). The use of this vaccine did not induce any tuberculin skin test reactivity and it reduced the mean tuberculous lung lesion score in challenged cattle. It was also reported that more animals had extra-thoracic spread of disease than BCG-vaccinated animals, indicating that immunization with some vaccines may exacerbate disease (Buddle *et al* 2003).

Novel immunostimulants that have particular application in stimulating a T_H1-dominated cytokine profile, are oligodeoxynucleotides (ODN) containing unmethylated CpG motifs. These CpG motifs help to control murine tuberculosis by non-specifically activating immune cells, but can also be used to enhance the efficacy of protein and DNA vaccines (Juffermans, Leeumans, Florquin, Verbon, Kolk, Speelman, van Deventer & van der Poll 2002). Reed *et al* (2003) also reported that the CpG and AS02 adjuvants have potential in TB vaccine studies. The addition of ODN with specific CpG motifs to a vaccine composed of *M. bovis* culture filtrate proteins and Polygen adjuvant was able to enhance IFN-γ responses to bovine PPD. These studies are in progress, and whether this vaccine will protect against BTB remains to be seen (Buddle *et al* 2003).

2.7.3 Killed micro-organisms

Killed mycobacterial vaccines are perceived to have certain advantages such as being safer than live attenuated vaccines, but the observed outcome from the use of these vaccine trials have been disappointing (Buddle 2001; Buddle *et al* 2003). In deer, killed BCG in oil produced strong tuberculin skin test reactivity, lymphocyte

transformation responses and high levels of antibody, compatible with a T_H2 type immune response whilst not being protective against bovine tuberculosis (Griffin, Mackintosh, Slobbe, Thomson & Buchan 1999). Killed *Mycobacterium vaccae* has been advocated for the immunoprophylaxis of tuberculosis in animals. Partial protection was showed in laboratory animals, where the lesions in vaccinated animals were less severe and they lived longer than non-vaccinated animals. (Stanford, Rook, Bahr, Dowlati, Ganapati, Ghazi, Saidi, Lucas, Ramu, Torres, Minh & Anstey 1999). The protective effects are considered to arise from stimulation of cellular immune responses to common mycobacterial antigens and from switching off the tissue-necrotizing effects.

Calves were vaccinated intradermally with killed *M. vaccae* and then challenged with live *M. bovis*, but showed no protection against BTB when compared to calves vaccinated with BCG (Buddel *et al*, 1995). Protection against *M. bovis* was induced when a combination of killed *M. vaccae* and live BCG was administered intranasally and intraconjunctivally to possums. This method of vaccination delivered greater protection compared to BCG vaccination alone (Skinner, unpublished). This raises the possibility that the efficacy of BCG might be enhanced by killed *M. vaccae.*

2.7.4 Live attenuated micro-organisms

The live attenuated micro-organism vaccine group refers to several strains of vaccines that were developed by different people using different growth media (see pages 19 – 21 on history of BCG vaccines). Ordinary BCG, modified BCG and attenuated *M. bovis* strains, are all part of the live attenuated micro-organism group of vaccines. These vaccines suffer from the disadvantage that they require extensive safety testing and live mycobacterial vaccines may induce positive tuberculin skin test reactions (Buddle *et al* 2000; Buddle *et al* 2003). Another factor to take into consideration is the increasing concern about the use of live vaccines in immune-compromised people, e.g. those with HIV infection (Reed *et al* 2003).

The live attenuated micro-organisms result in long-lasting CMI responses, which are necessary for protection against infection with intracellular bacteria such as *M. bovis* (Buddle *et al* 2003). BCG has been tested in a variety of species and administered via different routes, therefore it also produced different levels of protection across a wide range of doses. BCG has also been modified by gene deletion in an attempt to reduce skin test responses and to create a safer vaccine for immunocompromised individuals. The modified BCG is believed to induce a higher level of protection against *M. tuberculosis* in a guinea pig model than the parent BCG strain (Horwitz, Harth, Dillon & Maslesa-Galic 2000). The auxotrophic (requiring a specific organic growth factor not usually needed by parental strain) mutants of BCG have not been evaluated for protection against BTB in cattle (Buddle *et al* 2003). Several attenuated strains of virulent *M. bovis* were developed by chemical mutagenesis using a liquid culture with nitrosoguanidine. Two of these auxotrophic *M. bovis* strains were tested for protection of cattle against BTB, and resulted in fewer animals developing tuberculous lesions compared to the BCG-vaccinated and control groups (Buddle, Wards, Aldwell, Collins & de Lisle 2002). It is encouraging that the newly-derived attenuated *M. bovis* strains appear to perform even better than BCG.

2.8 BCG vaccines: history and development

A virulent *M. bovis* strain was isolated by Nocard from a heifer with tuberculous mastitis, and transferred to Institut Pasteur in 1901. This strain was destined to be the source of the many differing BCG substrains. All existing *M. bovis* BCG substrains originated form the BCG strain developed by Calmette and Guérin from 1908 – 1919 by 230 passages over glycerinated bile potato medium (Guérin 1980; Gheorghiu 1996). In the years following, a number of countries received BCG cultures from the Pasteur Institute, all propagating BCG in different ways, mainly by subculturing on potato or Sauton media (Osborn 1983). Therefore it is not surprising that different variants have emerged during the production of the BCG vaccine (Frappier & Panisset 1960; Osborn 1971). During the early development stages it was not possible to lyophilise BCG, therefore it continued to be grown in

much the same conditions that resulted in its attenuation, until the lyophilisation of BCG-Pasteur after 1173 passages in 1961 (Georghiu, Augier & Lagrange 1983).

Over the course of a few decades, each of these BCG vaccine laboratories developed its own daughter strain of BCG, typically named after the laboratory director, the city or country (Osborn 1983). After a large international congress in 1947, debating the future of BCG, these various substrains have been the objects of intense research ever since. At the time, more than 50 substrains were known to exist and results from clinical studies were compared. Many of these substrains are no longer in use (Oettinger, Jørgensen, Ladefoged, Hasløv & Andersen 1999). An important BCG substrain worth mentioning is Gothenburg, because it is the only known strain to have been kept exactly according to Calmette's instructions, rendering it the BCG strain closest to the original parent strain.

Among all the different BCG substrains currently produced, the following are in extensive use today: Connaught (Montreal), Danish, Glaxo (Merieux), Moreau, Pasteur, and Tokyo (Oettinger *et al* 1999; Behr 2002). All these strains show morphological, biochemical and immunological differences while markedly different levels of vaccine efficacy ranging from 0 – 94% have been reported for some of the substrains (Ponninghaus, Fine, Sterne *et al* 1992). Dietrich, Viret & Hess (2003) explained that it is difficult to evaluate the efficacy of the BCG vaccines, mainly due to the lack of comparability between different vaccination studies as a result of the use of different substrains, variable doses and immunization schedules as well as route of administration.

2.8.1 BCG Pasteur

The present BCG Pasteur strain was derived in 1961 from a single colony selected from 30 colonies that were examined. This colony yielded cultures that revealed properties most closely corresponding to the original description by Calmette, especially in terms of pigmentation (Georghiu *et al* 1983). Genomic comparisons indicate that BCG-Pasteur has undergone one single nucleotide polymorphism,

four deletions and two duplications between its first use in 1921 and lyophilisation in 1961 (Behr 2002). This strain, 1173P, was freeze-dried and the second batch is now the primary seedlot of the ”Paris or French strain” (Georghiu *et al* 1983). The 1173P Pasteur substrain was distributed to many other countries, some of which continue to produce BCG, but it is no longer used for BCG production in France (Oettinger *et al* 1999).

Historical and genetic data provide evidence that BCG strains have evolved since 1921, but what needs to be determined is whether these genetic changes will have an effect on their capacity to serve as anti-tuberculosis vaccines (Behr 2002). Based on different literature studies, one would expect that BCG strains derived from the ‘early’ BCG substrains to be more virulent and immunogenic, thus more protective than the ‘late’ BCG substrains. However, most of the ‘early’ substrains now in use, including BCG Moreau and Tokyo, have only been moderately immunogenic in animal studies, whereas some of the ‘late’ substrains like BCG Danish and Pasteur are some of the strongly immunogenic members of the BCG family (Jespersen 1971).

2.9 Review of publications on BCG vaccination in animals

BCG strains induce equal protection in some animal models, while other models suggest that some BCG strains give superior protection, and others markedly inferior levels of protection (Lagranderie, Balazuc, Deriaud, Leclerc & Gheorghiu 1996). A definite conclusion about efficacy is difficult to reach as varying methodologies were used in these studies, and it is not possible to identify a preferred BCG strain (Behr 2002).

2.9.1 Vaccination of mice

Mice (Family – *Muridae*) vaccinated with *M. bovis* BCG with or without either IL-12 or oligonucleotides containing cytidine phosphate guanosine (CpG ODN) adjuvants and challenged via the aerosol route 6 weeks post-vaccination with *M. tuberculosis* showed that vaccinated animals had a 1 – 2 log reduction in bacterial loads

compared to control mice (Freidag, Melton, Collins, Klinman Cheever, Stobie, Suen & Sederet 2000). Those vaccinated with IL-12 or CpG ODN had a 2 – 5 fold lower bacterial load than those vaccinated with BCG alone. Similarly, tuberculous granulomas in BCG-vaccinated mice were smaller than those in unvaccinated mice (Suazo, Escalera & Gallegos Torres, 2003).

2.9.2 Vaccination of guinea pigs

Results of BCG efficacy in guinea pigs (*Cavia porcellus*) are similar to those in other species. When challenged with live *M. bovis* by the aerosol route, vaccinated guinea pigs had fewer lesions and lower mycobacterial counts than non-vaccinated animals (Williams, Davies & Marsh 2000). BCG vaccination in guinea pigs was more effective against challenge with *M. bovis* than *M. tuberculosis* (Baldwin *et al* 1998). It is also suggested that the use of IL-12 and IL-2 as adjuvants might be useful in the reduction of mycobacterial counts in the lungs. Another important finding was that no organisms were recovered from the spleen of any of the guinea pigs vaccinated with the BCG Pasteur strain (Chambers, Williams, Gavier-Widen, Whelan, Hall, Marsh, Bloom, Jacobs & Hewinson 2000).

2.9.3 Vaccination of ferrets

Qureshi, Labes, Cross, Griffin & Mackintosh (1999) reported that there was a significant reduction in the number of gross tuberculous lesions among ferrets (*Mustela furo*) vaccinated orally with BCG (Pasteur 1173P2) compared to control animals. Fewer vaccinated ferrets had histologically detectable acid-fast organisms in the mesenteric lymph nodes as well as significantly fewer culture-positive retropharyngeal lymph nodes. The mean bacterial burden of the retropharyngeal lymph nodes of vaccinated ferrets was also significantly lower when compared to that in control animals.

Cross, Labes, Griffin & Mackintosh (2000) found that both intra-intestinal and systemic vaccination induced tuberculin-specific lymphocyte activity, but that only animals vaccinated by the subcutaneous route had reduced disease.

Subcutaneous vaccination in ferrets significantly reduced bacterial burdens, caused a decrease in disease severity and absence of gross lesions in the mesenteric lymph nodes (primary site), but it did not reduce the level of mesenteric lymph node infection. All ferrets in the above-mentioned experiments were challenged via the oral route to simulate natural infection.

2.9.4 Vaccination of badgers

When badgers (*Meles meles*) were vaccinated intradermally with BCG, they showed significantly higher peripheral blood mononuclear cell (PBMC) counts when stimulated with PPD as compared to unvaccinated animals (Southey, Sleeman, Lloyd, Dalley, Chambers, Hewinson & Gormley 2001). However, lower T-lymphocyte responses were experienced when compared to what was seen in cattle where similar dosage rates and routes were used (Carpenter, Fray & Gormley 1997), as well as in badgers with natural *M. bovis* infection (Dalley, Chambers, Cockle, Pressling, Gavier-Widen & Hewinson 1999).

Stuart, Mamood, Stanford & Pritchard (1988) did a study where the protective effect of BCG (Glaxo) was evaluated in a captive badger population. An intradermal BCG injection was reported to be safe without causing hypersensitivity reactions or disease, and no mycobacteria were excreted by the badgers and transmitted to in-contact animals. When vaccinated animals were subsequently challenged intradermally with live *M. bovis*, the injection sites healed faster, the badgers lived longer, and mycobacterial shedding was less than that in the control animals. Although small numbers of badgers were involved, it appeared that BCG vaccination enhanced CMI responses, delayed shedding of tubercle bacilli, and prolonged the lives of the badgers (Southey *et al* 2001).

2.9.5 Vaccination of possums

Different success rates were reported when possums (*Trichosurus vulpecula*) were vaccinated with the same BCG strain (Pasteur 1173P2) via various routes (Aldwell, Keen, Stent, Thomson, Yates de Lisle & Buddle 1995a). Tuberculous lesions in the

lungs, severity and extent of tissue damage and the number of acid-fast bacilli within the lesions were significantly higher in unvaccinated animals and animals vaccinated via the intragastric route (Aldwell, Pfeffer, de Lisle, Jowett, Heslop, Keen, Thomson & Buddle 1995b). Subcutaneous and intratracheal vaccination resulted in significant protection against pulmonary disease, while the injection of BCG into the duodenum caused a decrease in the development of tuberculous lesions in the infected animal. However, these results indicate that there was not complete prevention of infection (Buddle, Aldwell, Keen, Parlane, Yates & De Lisle 1997).

The ability of the BCG vaccine to protect possums against BTB infection seemed to wane over a longer period of time. The immune response was much stronger at two months post-vaccination than when measured at twelve months post-vaccination (Corner, Buddle, Pheiffer & Morris 2001). Revaccination at short intervals is also said to enhance protection. Protection against infection was significantly better when possums were vaccinated at weekly intervals rather than only once or twice at six-weekly intervals (Corner, Buddle, Pheiffer & Morris 2002). When necropsied, the lung weight, proportion of microscopic lung lesions, and bacterial counts of the spleen were significantly different in vaccinated possums when compared to unvaccinated animals.

2.9.6 Vaccination of monkeys

Monkeys have been used in several studies to test the efficacy of the *M. bovis* BCG vaccine, because they develop a disease that is similar to that observed in humans. The aerosol and intravenous (IV) routes of BCG vaccination have been equally good in protecting rhesus monkeys (*Macaca mulatta*) against low dose aerosol challenge, when the number of viable mycobacteria recovered, was used to determine the level of protection against infection and the development of the disease (Barclay, Anacker, Brehmer, Leif & Ribi 1970; Barclay, Busey & Delgard 1973). Intravenous vaccination with either the attenuated *M. bovis* strain or BCG vaccine also protected against *M. tuberculosis* (Ribi, Anacker & Barclay 1971).

Janicki, Good Minden, Afronti & Hymes (1973) reported that strong delayed-type hypersensitivity (DTH) reactions developed within 4 weeks after intradermal, IV and aerosol routes of BCG vaccination.

Vaccination has not always been that successful. Chaparas, Good & Janicki (1975) reported that although vaccination delayed the development of lesions, both vaccinated and non-vaccinated animals had TB lesions by 12 weeks after challenge. Similar results were found in cynomolgus monkeys (*Macaca fascicularis*) where high mortality was observed in all groups of experimental animals after 32 weeks of infection (Walsh, Tan & de la Cruz 1996). McMurray (2000) reported that very few studies of experimental tuberculosis in primates have been published. However, primates exhibit antigen-induced T lymphocyte reactivity both *in vitro* and *in vivo*, and therefore it is believed that BCG vaccination should be able to protect them effectively.

2.9.7 Vaccination of deer

A series of studies have been carried out in red deer (*Cervus elaphus*) to identify critical variables such as dose, route, boosting and formulation, all influencing the efficacy of BCG vaccines. Griffin *et al* (1999) showed that significant protection against infection and disease were obtained following boosting with two low doses (5×10^4cfu) or moderate doses (5×10^7cfu) of live (freshly cultured and lyophilized) BCG Pasteur 1173P2. Similar levels of protection were obtained regardless of whether the vaccines were administered subcutaneously (SC) or via the tonsilar route (Griffin 2000). Higher doses of live BCG (5×10^8cfu) delivered inferior levels of protection, while killed vaccine in mineral-oil adjuvant did not protect against infection or disease, although it caused a strong cellular immune response. Treatment with dexamethasone prior to vaccination with live BCG completely prevented any protective response (Griffin *et al* 1999).

Conclusions from studies done in red deer are that primary vaccination was effective in protecting against disease, but that boosting was essential in

preventing establishment of infection. It was also observed that the lower dosages, which protected against disease, did not evoke any DTH responses nor lead to any unacceptable side effects (Griffin *et al* 1999).

2.9.8 Vaccination of cattle

The earliest studies of BCG vaccination in cattle were done during the late 1940s. It was found that the immune response of calves vaccinated with BCG was strong after a challenge with virulent strains of *M. bovis* (Francis 1947). However, when animals were exposed to natural conditions of infection, ambiguous results were reported. Some researchers found fewer lesions in vaccinated animals, while others reported that there was no difference between vaccinated and non-vaccinated animals. Relatively high doses of BCG ranging from 10^7 to 10^9 colony forming units (cfu) of live BCG were used in these trials (Francis 1958). Vaccine studies done in Great Britain from 1940 to 1950 revealed that fewer vaccinated animals within a BTB infected herd developed lesions, when compared to non-vaccinated animals (O'Reilly & Daborn 1995).

Elwood and Waddington (1972) reported on two BCG studies that were done on cattle in Malawi. They found that BCG vaccinated cattle were more resistant to the development of tuberculous lesions and that 50% of controls and only 16% of vaccinated animals were condemned at the abattoir. It is also reported that a single subcutaneous (SC) inoculation of BCG induced protection against the development of TB lesions in animals experimentally infected with virulent *M. bovis*. It seems that vaccination decreases the contagiousness of infected animals by reducing the size of lesions and lowering the burden of mycobacteria, rather than preventing infection (Suazo *et al* 2003). More recently, it has been suggested that lower doses of BCG may preferentially stimulate the appropriate immune response for protection against mycobacterial infections (Bretsher 1992; Buddle 2001).

Buddle, de Lisle, Pfeffer and Aldwell (1995a) reported that low doses of BCG (10^4 to 10^6) administered subcutaneously could significantly protect calves against the

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development of lesions after *M. bovis* challenge. Another study showed that subcutaneous as well as intratracheal vaccination with BCG Pasteur 1173P2 protected against the development of lesions when cattle were experimentally infected with *M. bovis* (Buddle *et al* 1995b). However, vaccination with killed *M. vaccae* did not induce protection against *M. bovis* infection. Wedlock *et al* (2000) reported similar findings where vaccinated cattle had significantly lower lung lesion scores and mycobacterial counts than non-vaccinated cattle. The route of vaccination also seems to play a role in protection, and an important feature of intra-mammary vaccination was that antigen recognition also occurred at sites distant from the mammary gland (Nonnecke, Elsken & Kehrli 1986).

Vaccination with BCG in cattle is not always effective and it was reported in more than one study that vaccinated and non-vaccinated calves had a similar risk for condemnation when slaughtered (Berggren 1981). It is also postulated that the efficacy of BCG vaccination diminishes with the age of the animal (Berggren 1977). Despite several reports of BCG failure (Maes 1999; Agger & Andersen 2002) and all the excitement associated with novel TB vaccines, studies in the laboratory have shown that DNA and recombinant vaccines provided less protection against BTB infection and the development of lesions in cattle, than the BCG vaccine (Suazo *et al* 2003).

2.10 Applications of and expectations for TB vaccines

The three most important recipient groups to be considered for the development and testing of TB vaccines are humans, domestic animals and wildlife reservoirs. Different objectives need to be accomplished when these groups are considered (McMurray 2001). Although important, the issue of side-effects would impact much less on the regulatory approval of TB vaccines in animals, as compared to human TB vaccines. The most important current interest is the application of vaccination in domestic animals and wildlife.

A prophylactic vaccine would be considered effective in wildlife reservoirs if it can prevent transmission between infected individuals and susceptible contacts. Another important issue is the ability of the vaccine to prolong survival of the animal and reduce the effects of clinical disease, such as weight loss, fever and respiratory distress. These factors might be intrinsically desirable in those instances where rare or otherwise valuable wildlife reservoirs are threatened. While the success of mucosal delivery might be desirable in all three target groups for practical reasons, it is likely to be essential in wildlife reservoirs. The reason being that the only feasible approach to widespread application of the vaccine in especially nocturnal and burrowing animals will be self-administration via the oral and respiratory routes with the use of bait (Buddle, Skinner & Chambers 2000b).

The criteria in domestic animals, seems to be different. Due to the economic consequences of TB in domestic livestock, especially in countries where BTB is a controlled disease, a significantly higher burden is placed on such vaccines. The only goal for vaccination as a primary disease control strategy in domestic species will be to prevent infection (McMurray 2001). In domestic animals it would be highly desirable, if not essential, that TB vaccines do not interfere with routine diagnostic tests. This feature might be less important in wildlife reservoirs, because they are not regularly tested (McMurray 2001). To date, no vaccine has prevented the establishment of infection with virulent mycobacteria in any animal species (McMurray 2001).

2.11 Testing the safety of vaccines

All veterinary biological products administered to animals in South Africa must be tested for safety in the field before authorisation for marketing is granted. The purpose of the safety data of veterinary biologicals is to prove that the use of the product according to its label, does not pose any danger to the life, general well-being or production potential of the animal to be vaccinated. The evaluation of the safety of the use of the product is also of prime importance to human health to ensure that no harmful residues are present in animals that are destined for human

consumption. Field safety tests should be done in the target species and could be combined with field efficacy tests (OIE, 2000; EMEA, 2002).

The live BCG Pasteur vaccine was proved to be safe in all animal species to which it was administered. No side effects were observed and BCG was also not cultured from lymph nodes draining the original vaccination sites (Griffin *et al* 1995). No abnormal swelling or development of abscesses were seen at the vaccination site, and BCG was not transmitted to in-contact animals (Buddle *et al* 1995b). The different dosage rates seemed affect the efficacy of the vaccine rather than the safety of the product (Aldwell *et al* 1995; Buddle *et al* 1995a; Griffin *et al* 2001).

2.12 Testing the efficacy of vaccines

The efficacy of biologicals for prevention of disease in animals is almost always demonstrated by statistically valid vaccination-challenge studies in the host animal. These tests should be performed under controlled conditions starting with seronegative animals. Challenge should be carried out with an acceptable strain of the pathogen with a suitable level of pathogenicity (USDA 1999). The efficacy of the vaccine is firstly dependent on the quality of the product, which is determined by the nature and quality of the starting materials and the manufacturing process (OIE 2000; EMEA 2002).

Fewer lesions at the macroscopic and microscopic level, decreased bacterial burdening in target organs (lymph nodes, lung, spleen, liver) as well as prevention of bacterial dissemination beyond the focus of initial infection, are probably the three most important issues surrounding the efficacy of vaccines against TB in animals. Macroscopic lesions concern the number, size and location of tubercles and the amount of normal tissue compromised. The bacterial burden refers to the number of acid-fast organisms present in histopathological sections or impression smears or quantitative culture of tissues. Prevention of mycobacterial dissemination refers to the anatomical restriction of bacilli to the point of entry, thereby preventing extrapulmonary disease (Aldwell *et al* 1995; Buddle *et al* 1997;

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Buddle *et al* 2000b). However, it is now known that none of the above-mentioned parameters truly reflect the ability of the vaccine to prevent transmission of infection.

Different routes of administration as well as different dosages of the vaccine contributed to the variation in the efficacy observed (Aldwell *et al* 1995; Buddle *et al* 1995; Griffin *et al* 2001). It is suggested that lower doses of BCG may preferentially stimulate immune responses for protection against *M. bovis* (Buddle *et al* 1995). The administration of multiple rather than single vaccinations also influence the efficacy of the BCG vaccine (Griffin 2000).

2.13 Rationale for the choice of BCG-Pasteur 1173P2

The existing BCG vaccine is cheap to produce, can be administered via a number of routes, is safe, relatively stable and was originally derived from *M. bovis.* The vaccine is also readily available and currently in use in several countries (Buddle 2001). Where similar dosages and routes of administration were applied, reliable results of protection against disease, reduction in lesions and lowering of mycobacterial counts were observed. Data on several vaccine studies involving the administration of BCG-Pasteur 1173P2 are available for different species (Aldwell *et al* 1995; Buddle *et al* 1995; Griffin *et al* 2001).

Extensive studies have been carried out to evaluate the efficacy of BCG vaccine (Pasteur 1173P2) in experimentally infected deer. High success rates have been reported in protection against disease, while booster vaccination was found necessary to protect the animals against the establishment of infection (Griffin 2000). In a study by Buddle *et al* (1995), where the same BCG strain was used to vaccinate calves, the results revealed that a single inoculation was successful in reducing lesion development and that significantly fewer calves where diseased compared to non-vaccinated animals. Similar levels of protection resulted from vaccination with either a low or medium dose of BCG administered subcutaneously and from vaccination with a medium dose via the intratracheal route. An absence

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of lung lesions in the *M. bovis* challenged animals as a result of intratracheal vaccination, may result in a reduction in the transmission of *M. bovis* between cattle. Thus far, no other 'new TB vaccine' has outperformed the use of the BCG vaccine in cattle (Suazo *et al* 2003).

2.14 Implementing the intratonsilar route to challenge experimental animals with live *Mycobacterium bovis*

Vaccine efficacy can only be determined if a proper infection model is in place. The studies of Mackintosh *et al* (1995) and Palmer *et al* (1999) showed, respectively, that macroscopic tuberculous lesions that developed in red deer and cattle after the intratonsilar infection method was applied, were indistinguishable from natural infection. Histopathological examination could also not differentiate between naturally occurring disease and lesions that developed due to intratonsilar infection. The intratonsilar route of infection is also regarded as the safest and most accurate method of administering an exact dose of live *M. bovis* to a specific organ or lymph node within the experimental animal, without causing possible environmental contamination (Griffin *et al* 1995).

The majority of lesions recorded in buffaloes in the KNP were confined to the lungs, thoracic lymph nodes and lymph nodes of the head and neck (Bengis *et al* 1996). Tuberculous lesions observed in the tonsils and retropharyngeal lymph nodes were of specific interest and importance to future research studies concerning BTB in buffaloes. Visibility of the tonsilar crypts in buffaloes can easily be achieved with the aid of a laryngoscope (pers. comm. M. Bush, Smithsonian Institution, USA 1999), which would make this challenge method possible.

A study where the efficacy of a vaccine in African buffaloes is to be determined thus needs to involve two separate stages. Firstly, an infection model needs to be established. The second stage comprises testing the efficacy of the BCG using the infection model.

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CHAPTER III

3 MATERIALS AND METHODS

3.1 Animals

3.1.1 Infection model group

Twenty-eight (28) buffalo calves, aged 12-24 months, were captured on a game farm free of bovine tuberculosis in the Phalaborwa district (24° 00, 79.8' South; 31° 03, 58.0' East) during November 1999. The animals were transported to the State Veterinary enclosures at Skukuza, Kruger National Park (24° 59, 39.9' South; 31° 35, 10.2' East) where they were kept for the duration of the experiment. The animals were divided at random into three groups, each group being housed in a separate enclosure. The control group of animals (n = 6) was kept distant from Group 1 (n = 11) and Group 2 (n = 11). Groups 1 & 2 were given different doses of *Mycobacterium bovis* colony forming units by intratonsilar injection on 31 January 2000 and were identified as the high dose (HD) and low dose (LD) groups thereafter.

3.1.2 BCG vaccine study group

Thirty calves varying in age from ten to twenty months, from five different buffalo herds selected for their known negative TB status, were captured in the northern districts (Figure 3.1) of the Kruger National Park. Buffalo calves were numbered as follows:

Malahlapanga (W): LM 1/z – LM5/z, LM7/z

Shipande (X): LM 8/z – LM12/z, LM14/z – LM16/z

Klopperfontein (Y): LM18/z – LM21/z, LM23/z – LM24/z

Tsombeyeni (V): LM 25/z to LM 31/z

Gadzingwe (AA): LM 32/z, LM 33/z & LM 35/z.

Fig 3.1: Distribution of buffalo herds in the northern and far northern districts of the Kruger National Park, where buffalo calves were obtained from herds V, W, X, Y & AA

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Each animal was ear-tagged and branded at the capture site. The calves were then transported to an enclosure near the Shingwedzi tourist camp where they were kept for 14 days until the IFN-γ assay and comparative intradermal test results were available. Thereafter they were transported to the enclosures at Skukuza. After 2 months in captivity they were divided at random into 2 groups of 15 animals each. All yearlings from the first group were vaccinated using a BCG vaccine, while the other calves served as non-vaccinated controls. The animals were kept together in a single enclosure (24° 59, 41.1' South; 31° 35 10.1' East). They were fed a mixture of teff hay and lucerne and had access to water ad lib.

3.2 Bacillus Calmette-Guérin (BCG) vaccine

The original seedstock of BCG was obtained from the Disease Research Laboratory, University of Otago, Dunedin (New Zealand), and was transported in fluid medium to the Tuberculosis Laboratory at the Onderstepoort Veterinary Institute, South Africa. Subcultures from the master seed were grown in 7H9 broth (Difco Laboratories, Detroit, USA) supplemented with 10% OADC (3.83g NaCl, 25g BSA, 15ml sodium oleate and 20ml of 50% glucose in 465ml of distilled water) and 0,05% Tween 80 (VWR International, Merck house, UK). Cultures were grown at 37°C, without shaking, to mid-logarithmic growth phase. Cultures were then washed three times and the resuspended bacteria were counted using a phase contrast microscope. The number of colony forming units (cfu) present was confirmed retrospectively using plate counts. Dosages of 1,7 x 10^5 cfu and 1,3 x 10^6 cfu were administered during the primary and booster vaccinations, respectively.

The vaccine was only administered to the 15 calves in the vaccine group from the BCG vaccine study. One millilitre of freshly cultured BCG (Pasteur strain 1173P2) vaccine was injected intramuscularly in the left rump, about 10cm behind the palpable wing of the ileum. The use of a specific site ensured that each animal could be checked for the development of abscesses at the site of vaccination. After

six weeks the second dose of vaccine was administered into the muscle at the same site.

3.3 The *Mycobacterium bovis* strain used for experimental intratonsilar infection

During a survey carried out in 1998 to determine the prevalence of bovine tuberculosis in buffaloes in the KNP, tissue specimens from animals suspected to suffer from BTB were cultured to confirm the diagnosis (Rodwell, Kriek, Bengis, Whyte, Viljoen, de Vos & Boyce 2001). One of the isolated strains was shown to be capable of causing the development of a variety of tuberculous lesions in African buffaloes (Bengis *et al* 1996). Mycobacterial isolates were identified using biochemical and PCR tests followed by RFLP (Restriction Fragment Length Polymorphism) characterisation of the *M. bovis* isolates (de Vos *et al*. 2000). One *M. bovis* isolate (case no. KNP 182) classified as representative of the dominant KNP genotype ZA-01 (de Vos *et al*, 2000), was selected for use as the challenge strain for these trials. Subcultures of this *M. bovis* isolate had been stored at –20°C on Lowenstein-Jensen slopes containing pyruvate.

For preparation of the different inocula used for challenge, growth from fresh subcultures was carefully suspended in saline containing 0,5% Tween 80 on the day of the experimental infection. The concentration of bacteria was adjusted by counting under a microscope serial dilutions in a Neubauer counting chamber to 3×10^2 (low dose inoculum) and 3×10^4 (high dose inoculum) per 200μl, respectively. Special care was taken to avoid the presence of clumps in the inocula, by thorough pipetting of the suspension and examination under a microscope. Aliquots of each serial dilution were plated out in triplicate onto Lowenstein-Jensen medium with pyruvate for quality assurance. To avoid desiccation during the prolonged incubation at 37°C, petri dishes were sealed and placed in a humid chamber for 10 weeks. Plate counts were done after the incubation phase to determine the exact dose of live *M. bovis* instilled into the tonsilar crypt.

3.4 Method of intratonsilar infection

After being immobilized, the calves were kept in sternal recumbency during this procedure. Their heads were lowered to allow any excess saliva to drain before the suspension was instilled into the left tonsilar crypt. Their jaws were pulled apart, their tongues pulled to the left, and a 400mm laryngoscope was used to depress the base of the tongue. This made it possible to view the tonsilar crypts. Saliva that collected in the pharynx during the procedure was sucked out using a plastic tube, 500mm in length and 7,5mm in diameter, attached to a vacuum pump and collecting jar. This procedure ensured that the calf could breathe freely and maintained good visibility around the tonsil. A suspension of live *M. bovis* was carefully instilled into the left tonsilar crypt of the animal using a 1ml syringe with a 250 mm blunt needle taking care not to injure the surrounding mucosa. Whenever spillage of the suspension occurred or haemorrhage from the tonsilar crypts were observed, these events were recorded on the data sheet.

The animals in the infection model study were divided into 3 treatment groups:

High dose (HD): A 0,2ml suspension of 3×10^4 virulent bacteria of the KNP field strain was injected into the left tonsilar crypt.

Low dose (LD): A 0,2ml suspension of 3×10^2 virulent bacteria of the KNP field strain was injected into the left tonsilar crypt.

Control group: Saline (0,2ml) injected into the left tonsilar crypt.

Animals in the BCG vaccine study were all given the same dose (3×10^4 virulent bacteria of the KNP field strain) of live *M. bovis*, instilled into the left tonsilar crypt 4 weeks after administration of the booster vaccination.

3.5 Immobilization

A mixture of etorphine hydrochloride (M99; Novartis Animal Health) and xylazine (Chanzine 2%; Centaur, Bayer Animal Health) was used to immobilize the buffalo calves during the intratonsilar infection (Bengis & Raath 1993). Xylazine is a good

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muscle relaxant and facilitate opening the jaws of the immobilized calves. Throughout the rest of the study azaperone (Stresnil; Janssen) instead of xylazine was used as sedative.

The calves were immobilized once a month to be weighed and samples to be collected. Blindfolds were used, after immobilization to prevent excessive stress in the animals. The calves were placed in sternal recumbency for the duration of the aneasthetic period, to prevent regurgitation and bloating. Calves were placed on a tarpaulin stretcher that could be attached to a spring scale. The scale was lifted by a hydraulic crane suspending the calf, thus enabling its body mass to be recorded. Each animal was thoroughly examined for signs of weight loss, diarrhoea or any external injury. Treatment was administered if deemed necessary.

3.6 Monitoring general health, body mass and condition

The enclosures were checked three times a day to ensure availability of adequate food and water. The animals were visually examined daily for any signs of disease. Body mass could only be determined during handling sessions, but condition scores were carried out visually. Pooled faecal specimens were collected from the enclosures once a week, and individual specimens were collected from the rectum of each calf during the monthly handling sessions, to monitor their internal parasite levels. The McMaster method for individual and pooled samples was used to determine the number of roundworm eggs and coccidia oocysts per gram of faeces (Reinecke, 1973). Amprolium 30% soluble powder (Virbac, 15th Road, Halfway House) was added to the drinking at 10mg/kg/day for 5 days, assuming that the mean body mass of buffalo yearlings was 250kg and that each individual consumed about 25 liters of drinking water per day. This only done when coccidia counts increased to above 10 000 oocysts per gram of individual faecal material or 5 000 oocysts per pooled sample.

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3.7 Comparative intradermal tuberculin test

All the buffalo calves in the Infection Model study were tested with the comparative intradermal tuberculin test before the start of the trial and again at three months post infection. The buffalo calves in the BCG Vaccine study were subjected to the comparative intradermal tuberculin test three times during the vaccination trial. The first intradermal test was done at capture and only animals that tested negative were included in the trial. The second test was done 4 months after the intratonsilar challenge with live *M. bovis*. The last skin test was conducted 3 months later and two weeks prior to the day of slaughter.

The comparative intradermal tuberculin test was done by injecting 0,1ml of bovine purified protein derivative (PPD) and 0,1ml of avian PPD intradermally. The skin was examined for the presence of any lesions that would interfere with the test. Rectangular areas (30mm x 20mm) were shaved on either side of the neck and the skin thickness of each of the sites measured. Bovine PPD (0,1mg/ml) was injected intradermally in the centre of the clipped area on the left side and avian PPD (0,05mg/ml) on the right side of the neck using a McClintock syringe.

After a period of 72 hours had elapsed, the calves were immobilised and the skin at each of the two injection sites examined and measured. The increase in skin thickness of the avian PPD injection site was subtracted from that of the increase in skin thickness of the bovine PPD site to get a final result. A total increase in skin thickness of more than 4mm was regarded as positive, an increase of 2mm–4mm as suspect, and an increase of less than 2mm was classified as negative (Guidance document on bovine tuberculosis testing, control and eradication: National Department of Agriculture). In known infected herds such as the two experimental study groups all suspect and positive reactions were considered to be **positive**, while avian, equal and negative reactions were regarded as **negative**.

3.8 Nasal swabs

Sterile cotton swabs of about 20cm in length were used to collect nasal mucus specimens from each calf. The swab was gently pushed up the nostril towards the medial canthus of the eye and slowly rolled to collect the nasal mucus taking care not to contaminate the cotton tip with soil. After collection the swabs were labeled, frozen and sent to the TB Laboratory at the Onderstepoort Veterinary Institute for mycobacterial culture.

3.9 Necropsy procedure

Calves from the Infection Model were euthanased at 22 weeks post challenge, because the aim was to establish whether there would be a difference in the lesion score of animals infected with different dosages. Animals from the BCG study had to be housed in the same holding facilities and therefore the previous experimental animals had to be removed so that the enclosures could be cleaned and sprayed with disinfectant. The calves from the BCG vaccine study were euthanased at 34 weeks post challenge, to allow for two intradermal tuberculin tests to be performed within the study period.

After euthanasia with a saturated succinylcholine solution the calves were removed from the bomas and weighed; the carcass was lifted by a crane and hung by a hind leg above a drain; the throat was cut; and the carcass was left hanging until exanguinated. Thereafter a standard necropsy combined with routine meat inspection was done. Upon arrival at the abattoir, the heads were removed from the carcasses and were placed on hooks together with the pluck from the same carcass onto a semi-automated system. All the different parts of one carcass were clearly identified with cards carrying the slaughter number of each individual animal. The carcasses were eviscerated and the abdominal viscera placed on a stainless steel working bench, where a thorough examination of the reticulum, rumen, omasum, abomasum, small and large intestine as well as the kidneys could

be carried out. During the processing of the carcass, all the lymph nodes were removed for slicing and detailed examination.

The retropharyngeal lymph nodes of each animal were labeled and photographed. The lungs were palpated to detect any lesions and were then systematically sliced in thicknesses of 20mm to aid the visual detection of any lesions. Where lesions were found, specimens were collected for mycobacterial culture and histopathology, respectively. Four different cutting boards were labeled with the slaughter number of a carcass for the different lymph node groups namely head, thorax, abdomen and carcass. Specimens in eight sterile culture bottles were submitted for mycobacterial culture from each animal in the experiment.

A general set of specimens for each animal consisted of:

1. Specimens for mycobacterial culture:

   Left medial retropharyngeal lymph node
   Right medial retropharyngeal lymph node
   Left tonsil
   Right tonsil
   Head pool (parotid ln, mandibular ln.)
   Thorax pool (bronchial ln, mediastinal ln.)
   Abdominal pool (hepatic ln, gastric ln, mesenteric lnn, ileocaecal ln, rectal ln.)
   Carcass pool (prescapularis ln, popliteal ln, axillary ln, inquinal ln, mammary/scrotal lnn.)

2. Specimens for histopathology:

   Left retropharyngeal lymph node together with any other lymph nodes in which lesions were seen.

The following criteria were used to score the macroscopical lesions observed in every individual animal from both the Infection and BCG vaccine studies:

0: No visible lesions
1: Less than 50% of a single lymph node affected
2: More than 50% of a single lymph node affected, but not more than two lymph nodes involved
3: More than two lymph nodes involved, including tonsils, but no organs affected
4: Multiple lymph nodes involved, with pulmonary granulomas
5: Generalized tuberculosis

3.10 Laboratory tests

3.10.1 Blood sampling

During each immobilization, several blood samples were taken from each buffalo via jugular puncture with a 1,2 × 38mm Vacutainer® needle (Becton Dickinson Vacutainer Systems, B.P.37-39241, UK). Blood in heparin-containing tubes were collected for use in the IFN-γ test, blood in K_3 EDTA-containing tubes were collected for whole blood counts, blood smears and PCR, while serum was collected for ELISA testing and serum storage. The blood in the tubes (Becton Dickinson Vacutainer Systems Europe, Plymouth, PL67BP, UK) was mixed gently immediately after collection. The heparinised blood samples were taken to the laboratory for processing within six hours. Whole blood counts were conducted with a Coulter counter® (T890 Vet #, Beckman Coulter, Halfway House) within twelve hours after collection. Stored serum samples were kept at -20°C.

3.10.2 Interferon-gamma (IFN-γ) assay

The IFN-γ assay is a rapid assay on blood to measure cell-mediated immunity (CMI) for the diagnosis of BTB in cattle. The assay is based on the principle that lymphocytes in the blood of the *M. bovis* infected animal detect specific mycobacterial antigens present in the bovine purified protein derivative (PPD). This recognition involves the generation and secretion of the cytokine, interferon-

gamma (IFN-γ). Tuberculin PPD antigens are presented to lymphocytes in whole blood cultures and the production of IFN-γ from the stimulated T cells is detected using a monoclonal antibody-based sandwich enzyme immunoassay (EIA). Lymphocytes from uninfected cattle do not produce IFN-γ and hence the increase IFN-γ levels correlates with infection. The test was developed and patented by CSIRO, Australia.

In this research project the IFN-γ assay was applied as described by Michel *et al* (2000). *Mycobacterium fortuitum* PPD was added to the normal protocol to enhance sensitivity and specificity of the original gamma-interferon assay. The amount of IFN-γ in each plasma sample was estimated and the relative levels of IFN-γ detected used to indicate whether an animal was infected with *M. bovis*. The optical density of the reaction was measured at 450nm using an EL312e spectrophotometer (Bio-Tek Instruments, Inc. Highland Park, Box 998, Winooski). During the first test run, only the plasma samples where bovine-PPD was incorporated were tested. Samples with an optical density (OD) value of higher than 0,27 were regarded as bovine reactors, and therefore had to be retested. This involved screening the whole plasma set (bovine, avian, fortuitum and control) from each animal on one plate simultaneously, so that differences in optical density between reactions could be determined, enabling the operator to rule out any false positive reactors.

Different outcomes such as positive, equal reactor, multiple reactor, avian reactor, invalid reaction and negative are all possible when the IFN-γ assay is run. The standard interpretation of the IFN-γ assay used by the Onderstepoort Veterinary Institute (OVI) for African buffaloes is summarised in Table 3.10.2.

The validity of the assay was also tested against the following criteria: positive control OD > 0,8 and the negative control OD < 0,1. The following criteria were applied: all positive, suspect and MR results were regarded as **positive**, while negative, avian, equal and invalid results were regarded as **negative**.

Table 3.10.2: The criteria used for interpreting the IFN-γ assay results.

Outcome	IFN-γ test result	Subject to	Provided that
Positive	OD_{bovine} - OD_{avian} > 0.20	$OD_{fortuitum}$ – OD_{nil} < 0.15	OD_{nil} < 0.25
Equal reactor	OD_{bovine} and OD_{avian} > 0.30	Difference < 0.15	$OD_{fortuitum}$ and OD_{nil} both < 0.25
Multiple reactor	OD_{bovine} and OD_{avian} > 0.30	$OD_{fortuitum}$ - OD_{nil} > 0.15	
Avian reactor	OD_{avian} - OD_{bovine} > 0.20		$OD_{fortuitum}$ and OD_{nil} both < 0.25
Invalid reaction	$OD_{untreated\ control\ plasma}$ > 0.25		
Negative	All OD values < 0.30		

3.10.3 Histopathology

Specimens were collected and preserved in 10% buffered formalin, and were later prepared routinely for light microscopy by embedding them in paraffin wax. Sections cut to a thickness of 4 - 6µm were routinely stained with haematoxylin and eosin, and selected sections with the Ziehl-Neelsen acid-fast stain. The pathologist also reported on the activity of the lesions, and depending on the presence or absence of certain cellular components the lesions were considered to be either active or static.

3.10.4 Acid-fast organism (AFO) count

The Ziehl-Neelsen stained histopathology sections were examined microscopically to detect the presence of AFOs. The pathologist started on the left side of the slide and gradually moved down in narrow strips to cover the whole slide. The individual AFOs were counted and the following scoring system was applied:

0: No AFOs seen
1: <5 AFOs per slide
2: 5 – 10 AFOs per slide
3: 10 – 20 AFOs per slide
4: Multiple/Many per slide

3.10.5 Mycobacterial culture

Tissue samples and nasal swabs that were submitted to the TB Laboratory at the Onderstepoort Veterinary Institute (OVI) were cultured to detect the presence of mycobacteria. Fresh lymph node specimens were collected and a set of at least eight lymph node specimen bottles was submitted for mycobacterial culture for each experimental animal. The left and right tonsils were kept separate, as were the retropharyngeal lymph nodes. Whenever a lesion was found in an organ or a lymph node other than the left retropharyngeal, it was collected separately. Pooled samples of lymph nodes from the head, thorax, abdominal cavity and carcass were also submitted for mycobacterial culture. Only one specimen pool needed to culture positive to confer a positive BTB status to the animal.

Methods for isolation and identification of mycobacteria as described by Nel, Kleeberg and Gatner (1980) were used. Lesions were excised and homogenized. The homogenized tissue was divided in two parts, one was decontaminated with 2% HCl, and the other with 4% NaOH. The samples were left at room temperature for 15 minutes, then centrifuged at 1 650g. The supernatant fluid was discarded, and the sediments washed by centrifugation and then inoculated onto each of two tubes of:

- Löwenstein-Jensen (LJ) egg-based medium with glycerine
- LJ medium without glycerine
- LJ medium with 0,5 % pyruvate

The tubes were incubated at 37°C and observed for growth of colonies of acid-fast bacteria at 1 and 2 weeks, and then at 2-weekly intervals for 10 weeks. As soon as colonies of acid-fast bacteria were observed, they were sub-cultured onto the same medium, that supported primary growth. The isolates were then identified by:

- their growth characteristics
- their preference for LJ medium supplemented with pyruvate
- their sensitivity to thiopene carboxylic-acid-hydrazide
- the results of biochemical tests

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Cultures were negative for *M. bovis* if no colonies were observed after 3 months of incubation.

3.11 Statistical analyses

Statistical analyses were done by the data analyst J.M. Kruger, from the Scientific Services Department, Skukuza. Analysis of variance (ANOVA) was the method of choice for all statistical analyses performed on the data (Zar, J.H. 1974). As the response variables were all continuous variables and the researcher was interested to see whether there were any significant differences between the positive and negative individuals over the specified time period an ANOVA could be used. Standard methods were used to calculate sensitivity and specificity of test results.

The median is usually studied where asymmetrical distribution of data needs to be interpreted. In the case of these experiments, only one animal became clinically diseased and therefore the haematology data could be interpreted as if no outliers occurred. It was then decided to study the mean rather than the median, because the data was believed to be of normal distribution.

The sample sizes of the different study groups were too small to detect statistically significant differences between vaccinated and control animals. A power calculation based on binomial proportion responders was done where 10/14 control animals were positive so the proportion responders was 71%, and for the vaccinated group, 8/15 or 53% were negative. Based on this power calculation, one will need 40 animals in the control and 40 animals in the vaccinated group to be able to detect a 10% difference with a 0.8 power.

CHAPTER IV

4 RESULTS

4.1 *THE INTRATONSILAR INFECTION MODEL*

4.1.1 Body mass and condition

No differences were detected between the average daily weight gain of diseased and healthy animals or the different treatment groups. Diseased, refers to animals with a positive *M. bovis* culture result, while healthy animals had a negative result. A decline in average daily gain and a lower than expected mean body mass were observed in the low dose (LD) group during the last month of the trial.

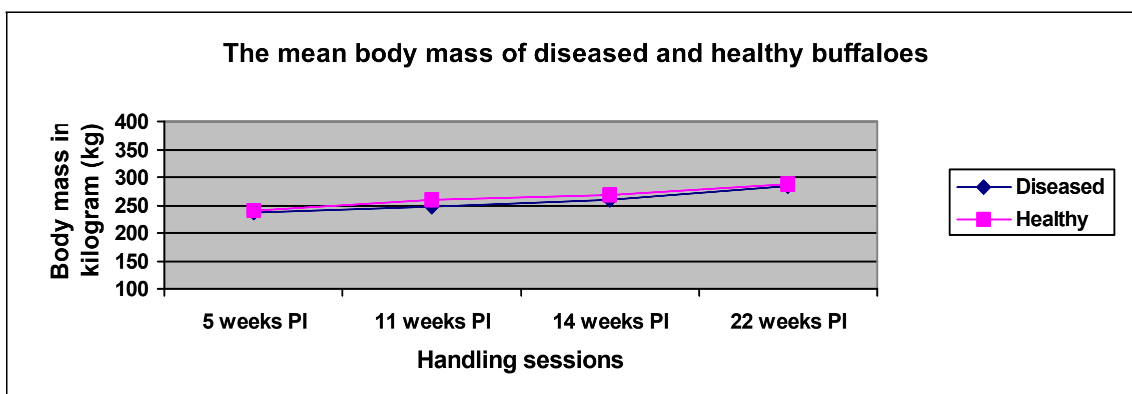

Fig 4.1.1.1: Gain in body mass of diseased and healthy buffalo calves during the course of the Infection Model.

One bull from the control group was eight months older than the rest of the calves and therefore had a higher body mass. This influenced the mean body mass of the control group, but had no effect on statistical analyses of the difference in body mass or average daily gain.

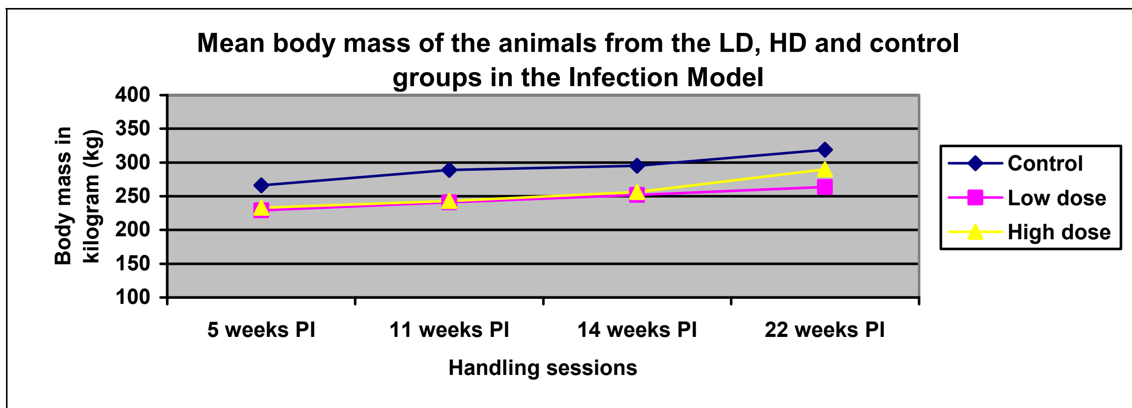

Fig 4.1.1.2: Gain in body mass of the buffalo calves from the LD, HD and control groups during the course of the Infection Model.

4.1.2 Mortalities

Two unexpected deaths that were not related to bovine tuberculosis occurred in calves from the high dose group. A heifer calf (LM 19) died 5 weeks after challenge due to respiratory failure during a handling session. Severe lung oedema and congestion were observed. The other animal, a bull calf (LM15), died 11 weeks after challenge due to an unexplained rupture of the cardiac muscle that caused a severe haemothorax. Although these animals did not live up to the date of euthanasia, meaningful results regarding tuberculous lesions, histopathology and *M. bovis* cultures were obtained.

4.1.3 Comparative intradermal tuberculin test

Two weeks prior to intratonsilar challenge, all animals were subjected to the comparative intradermal tuberculin test. All the experimental animals tested negative on this first intradermal tuberculin test.

The second intradermal tuberculin test was conducted 11 weeks post challenge. All the HD group animals (9/9) tested positive, while only 6/11 of the LD group animals reacted positively to the test. None of the control animals showed any reaction to the second test.

Table 4.1.3: Results of the intradermal tuberculin tests conducted 11 weeks after live *Mycobacterium bovis* challenge

Number	ITT[1] result	Increase in skin thickness	*M. bovis* culture	Reaction at injection site and comments
Controls				
LM 4	-	None	-	
LM 5	-	None	-	
LM 8	-	None	-	
LM 9	-	None	-	
LM 18	-	None	-	
LM 26	-	None	-	
Low dose				
LM 1	+	15,6mm	+	Oedema
LM 2	-	None	-	
LM 7	-	None	-	
LM 12	-	None	-	
LM 14	Suspect	2,8mm	-	Mild oedema
LM 17	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable[2]
LM 21	-	None	+	(False negative)
LM 22	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM 23	-	None	-	
LM 24	+	4,2mm	-	Mild oedema (False positive)
LM 27	Suspect	3,3mm	-	Mild oedema
High dose				
LM 3	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM 6	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM 10	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM 11	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM13	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM 16	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable
LM 20	+	17,2mm	+	Oedema
LM 25	+	4,9mm	+	Mild oedema
LM 28	+	> 33mm	+	Oedema, hyperaemia, necrosis, immeasurable

[1] **ITT:** This abbreviation for the comparative intradermal tuberculin test is only used in the tables included in this dissertation and is not accepted in general literature where bovine tuberculosis is concerned.

[2] **Immeasurable:** This refers to the inability of the caliper to measure the increase in skin thickness, due to oedema of such extent that the skin fold cannot fit into the space between the two legs of the caliper.

The immeasurable increases in skin thickness were also associated with sero-fibrinous exudate and necrotic foci of the skin at the bovine-PPD injection site. The majority of these skin reactions showed peripheral hyperaemia, were oedematous, and painful on palpation, even while the calves were immobilized. False positive and negative reactions only occurred in animals from the low dose group.

4.1.4 Necropsy results

The two calves from the HD group died before completion of the study as a result of disease conditions not related to TB. One animal died 5 weeks after infection and no macroscopic tuberculous lesions were present in any of the lymph nodes. No enlargement of the lymph nodes was noticed in the second animal that died 11 weeks post infection. However, a mild to moderate, multifocal to diffuse caseo-necrotic lymphadenitis of the left retropharyngeal lymph node was present in this animal. One animal from the LD group had a severe peritonitis because of a puncture wound in the reticulum due to a Dan-Inject dart that was swallowed during the previous handling session. No BTB-related lesions were observed in this animal.

In the rest of the experimental animals, lesions in the lymphoid tissues were seen primarily in the left retropharyngeal lymph nodes. Macroscopic lesions were seen in 4/11 LD animals and 9/11 HD animals. The lesions varied from pinpoint foci of caseous necrosis with a mild lymphadenitis to a severe, multiple to confluent, caseo-necrotic lymphadenitis. Lesions in animals of the low dose group were generally less extensive. Severe enlargement of the lymph nodes or disruption of the normal structure were not recorded for animals in the LD group.

The lesions of animals in the HD group were more extensive and a difference in size between the left and right retropharyngeal lymph nodes was commonly observed. Although some individual differences occurred, caseo-necrotic lesions with central calcification were generally found throughout the retropharyngeal

lymph nodes of animals in the HD group. Disruption of the normal structure of the lymph nodes was a more common finding in animals from this group.

Secondary lesions developed in only 2 animals (LM1 and LM10 from the LD and the HD groups, respectively) and both animals had lesions in the thoracic lymph nodes. LM 10 was the most severely diseased animal in the Infection Model with an almost ten-fold increase in the size of the affected left medial retropharyngeal lymph node. Almost 80% of the lymphoid tissue of the left retropharyngeal lymph node was replaced by a caseous, necrotic, granulomatous inflammatory reaction. While calcification was quite pronounced in some parts of the affected lymph node, a central focus of liquefaction was also present.

Figures 4.1.4.1 – 4.1.4.2 indicate the variety of lesions observed in experimental animals from the different treatment groups. All lymph nodes represented within these figures are left retropharyngeal lymph nodes.

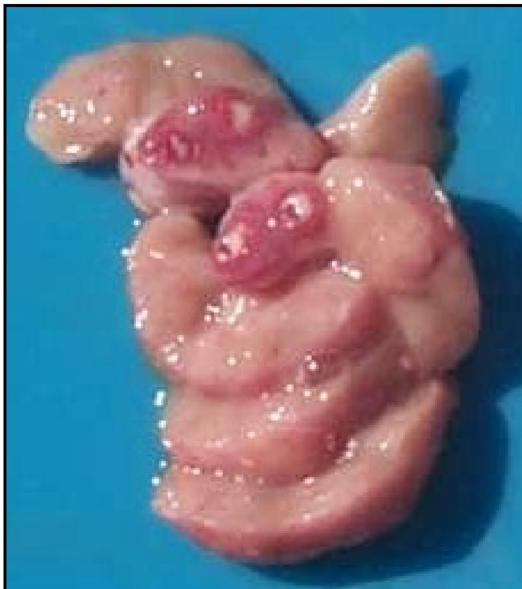

Fig 4.1.4.1: Two small foci of caseo-necrotic granulomas with a zone of hyperaemia. These lesions were more commonly seen in animals from the LD group.

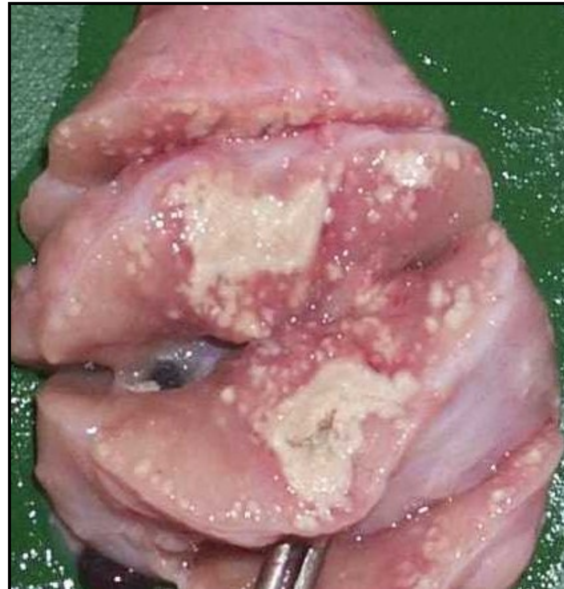

Fig 4.1.4.2: Caseo-necrotic granulomatous lymphadenitis affecting more than 50% of the lymph node as well as the presence of some liquefaction.

Table 4.1.4: The number of animals with macroscopic tuberculous lesions and the sum of the grading of the lesions observed in experimental animals from the different treatment groups in the Infection Model

Group	Number of animals with macroscopic tuberculous lesions						Grading
	Tonsil	**Left retro. In.**	**Head Inn.**	**Thorax Inn.**	**Lung**	**Other**	
Control n = 6	0	0	0	0	0	0	0
Low dose n = 11	0	4	0	1	0	0	8
High dose n = 11	0	9	0	1	0	0	17

As is indicated in Table 4.1.4, the sum of the grading score of the macroscopic tuberculous lesions observed in the lymph nodes of the HD group was twice as high as the total score for affected lymph nodes of animals from the LD group. Individual lesion score results can be viewed in Annexure I: Table 7.

4.1.5 Laboratory tests

4.1.5.1 Haematology

No differences at the 95% confidence level could be detected between the haematology results of diseased and healthy animals in the Infection Model, or between the animals from the different treatment groups. Individual results and statistical analyses are presented in Annexure I: Tables 6.1 – 6.4 and III: Study 1.

4.1.5.2 Interferon-gamma assay (IFN-γ)

Antigen-specific reactions can be viewed in the raw data. In experimental animals known to be challenged with live *M. bovis*, all positive, multiple reactor (MR) and suspect results for the IFN-γ assay were regarded as positive. All other reactions were regarded as negative results. The sensitivity and specificity of the IFN-γ assay

used in the Infection Model compared well to recorded results of the IFN-γ when used in domestic cattle.

Table: 4.1.5.2: The IFN-γ assay results at specific times after intratonsilar infection throughout the duration of the study

Number	Day 0	5 weeks	11 weeks	14 weeks	21 weeks	Final result
Control						
LM 4	-	-	-	-	-	Negative
LM 5	-	-	-	-	-	Negative
LM 8	-	-	-	-	-	Negative
LM 9	-	-	-	-	-	Negative
LM 18	-	-	-	-	-	Negative
LM 26	-	-	-	-	-	Negative
Low dose						
LM 1	-	-	-	+	+	Positive
LM 2	-	-	-	-	-	Negative
LM 7	-	-	-	-	-	Negative
LM 12	-	-	+	-	-	Positive
LM 14	-	-	-	-	-	Negative
LM 17	-	-	+	+	+	Positive
LM 21	-	-	-	-	+	Positive
LM 22	-	-	+	+	+	Positive
LM 23	-	-	-	-	-	Negative
LM 24	-	-	-	-	-	Negative
LM 27	-	-	-	+	-	Positive
High dose						
LM 3	-	-	+	+	+	Positive
LM 6	-	+	+	+	+	Positive
LM 10	-	-	+	+	+	Positive
LM 11	-	+	+	+	+	Positive
LM 13	-	+	+	+	+	Positive
LM 15	-	+	+	N/A	N/A	Positive
LM 16	-	-	-	+	+	Positive
LM 19	-	+	N/A[1]	N/A	N/A	Positive
LM 20	-	-	+	+	+	Positive
LM 25	-	+	-	+	+	Positive
LM 28	-	+	+	+	+	Positive

[1] **N/A:** No result available for assay due to death of animal before conclusion of the study.

4.1.5.3 Histopathology

Specimens from the retropharyngeal lymph nodes of the animals in the LD group were submitted for histopathology. In four of eleven specimens lesions consistent

with those of a mycobacterial infection were seen. In the HD group lesions were present in 9/11 animals. One animal (LM19) without macroscopic lesions had a granulomatous lymphadenitis on histopathology, while the histopathology section from the other animal (LM16) without macroscopic lesions, showed no inflammatory reaction or infection with mycobacterial organisms. Specimens of thoracic lymph nodes from only two animals (LM1 & LM10), from the LD and HD groups, respectively, were submitted for histopathology. Both animals had pinpoint foci of caseous necrosis in the mediastinal lymph nodes; histopathological examination revealed a granulomatous inflammatory reaction consistent with that caused by a mycobacterial infection.

The general histopathological features were those of multifocal areas of necrosis with central calcification and a peripheral accumulation of epithelioid macrophages and multinucleated giant cells. Mycobacteria in the stained positive with Ziehl-Neelsen stain, and were mostly present in the cytoplasm of the giant cells.

4.1.5.4 Acid-fast organism (AFO) count

The acid-fast organism (AFO) count was higher in the high dose group than in the low dose group. All the animals from the low dose group with positive histopathology results had < 5 AFOs, while the animals from the high dose group had a wider spectrum of AFO counts that ranged from 0 to multiple AFOs.

4.1.5.5 *Mycobacterium bovis* culture

If no mycobacterial growth was observed after 3 months of incubation, the cultures were regarded as negative. Bacterial growth was seen after 2 weeks in most of the specimens sent for bacterial isolation and *M. bovis* could be identified after 6 weeks. *Mycobacterium bovis* could be isolated from all the left retropharyngeal lymph node cultures of the animals from the HD group while 6/11 animals from the LD group had positive left retropharyngeal lymph node culture results. *Mycobacterium bovis* was not isolated from any of the lymph nodes collected from the control group.

Table 4.1.5.4: The histopathology findings and the acid-fast organism counts in the lymph nodes removed from the experimental buffalo calves of the Infection Model at necropsy.

Number	Lymph node	AFO count per slide	AFO score	Histopathology result
Control				
LM 4	L. Retropharyngeal	0	0	-
LM 5	L. Retropharyngeal	0	0	-
LM 8	L. Retropharyngeal	0	0	-
LM 9	L. Retropharyngeal	0	0	-
LM 18	L. Retropharyngeal	0	0	-
LM 26	L. Retropharyngeal	0	0	-
Low dose				
LM 1	L. Retropharyngeal	< 5	1	+
	Mediastinal	0		+
LM 2	L. Retropharyngeal	0	0	-
LM 7	L. Retropharyngeal	0	0	-
LM 12	L. Retropharyngeal	0	0	-
LM 14	L. Retropharyngeal	0	0	-
LM 17	L. Retropharyngeal	< 5	1	+
LM 21	L. Retropharyngeal	< 5	1	+
LM 22	L. Retropharyngeal	< 5	1	+
LM 23	L. Retropharyngeal	0	0	-
LM 24	L. Retropharyngeal	0	0	-
LM 27	L. Retropharyngeal	0	0	-
High dose				
LM 3	L. Retropharyngeal	0	0	+
LM 6	L. Retropharyngeal	< 5	1	+
LM 10	L. Retropharyngeal	0	0	+
	Mediastinal	0		+
LM 11	L. Retropharyngeal	0	0	+
LM 13	L. Retropharyngeal	Multiple AFOs	4	+
LM 15	L. Retropharyngeal	< 5	1	+
LM 16	L. Retropharyngeal	0	0	-
LM 19	L. Retropharyngeal	10 to 20	3	+
LM 20	L. Retropharyngeal	Multiple AFOs	4	+
LM 25	L. Retropharyngeal	0	0	-
LM 28	L. Retropharyngeal	10 to 20	3	+

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4.1.6 Summary of the final results from the experimental infection

From the summarized data of the Infection Model in Table 4.1.6 it can be seen that not all animals challenged with live *M. bovis* yielded positive culture results. Similarly even culture positive animals did not always test positive when the other laboratory tests were applied.

Table 4.1.6: A summary of the results obtained from the various field and laboratory tests in the experimental animals of the Infection Model.

Nr.	Gender	Skin test	γ-IFN	Macroscopic lesion	Histopathology	Culture
Controls						
LM 4	M	-	-	None	Neg. (0 AFO)	-
LM 5	M	-	-	None	Neg. (0 AFO)	-
LM 8	F	-	-	None	Neg. (0 AFO)	-
LM 9	F	-	-	None	Neg. (0 AFO)	-
LM 18	F	-	-	None	Neg. (0 AFO)	-
LM 26	M	-	-	None	Neg. (0 AFO)	-
Low Dose						
LM 1	M	+	+	L. retro & mediast. Inn.	Pos. (<5 AFO)	+
LM 2	M	-	-	None	Neg. (0 AFO)	+
LM 7	F	-	-	None	Neg. (0 AFO)	-
LM 12	F	-	-	None	Neg. (0 AFO)	-
LM 14	F	+	-	None	Neg. (0 AFO)	+
LM 17	F	+	+	L. retro ln.	Pos. (<5 AFO)	+
LM 21	F	-	+	L. retro ln.	Pos. (<5 AFO)	+
LM 22	M	+	+	L. retro ln.	Pos. (<5 AFO)	+
LM 23	M	Equal	-	None	Neg. (0 AFO)	-
LM 24	F	+	-	None	Neg. (0 AFO)	-
LM 27	M	+	-	None	Neg. (0 AFO)	-
High Dose						
LM 3	F	+	+	L. retro ln.	Pos. (0 AFO)	+
LM 6	M	+	+	L. retro ln.	Pos. (<5 AFO)	+
LM 10	F	+	+	L. retro & mediast. Inn.	Pos. (0 AFO)	+
LM 11	F	+	+	L. retro ln.	Pos. (0 AFO)	+
LM 13	F	+	+	L. retro ln.	Pos. (multiple)	+
LM 15	M	N/A[1]	+	L. retro ln.	Pos. (<5 AFO)	+
LM 16	M	+	+	None	Neg. (0 AFO)	+
LM 19	F	N/A	+	None	Pos. (10-20 AFO)	+
LM 20	M	+	+	L. retro ln.	Pos. (multiple)	+
LM 25	M	+	+	L. retro ln.	Neg. (0 AFO)	+
LM 28	M	+	+	L. retro ln.	Pos. (10-20 AFO)	+

[1] **N/A:** No result available for assay due to death of animal before conclusion of the study.

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4.2 *THE BCG VACCINE STUDY*

4.2.1 General health, body mass and condition

Two weeks after the booster vaccine was administered, several animals showed signs of diarrhoea. Faecal analysis indicated severe coccidiosis and blanket treatment with Amprol 30% (extra-label use) was repeated three times at one week intervals.

Seven weeks post intratonsilar challenge several animals were observed with saliva and foam dripping from their oral cavities. Most of the calves were reluctant to eat and favoured soaking their mouths in the water troughs. Ulcerative lesions were seen on the dorsal and lateral surfaces of the tongue and in some calves also on the dental pads and hard palate. Vaccine and control animal groups were equally affected. Epithelial samples from the oral cavity and oropharynx submitted for foot-and-mouth disease virus isolation tested positive. All the serum samples collected from the experimental animals and tested for foot-and-mouth disease virus with the ELISA were serologically positive. Body condition decreased dramatically within one week after the appearance of clinical signs. Larger animals weighing more than 270kg lost between 24kg to 43kg per animal. The smaller animals seemed to be somewhat less affected with some losing only 10kg while others lost no body mass at all. Loss in body mass was recorded for up to two months after the foot-and-mouth disease outbreak. Although two animals presented with interdigital vesicular lesions of the front feet, no lameness was recorded during this foot-and-mouth disease outbreak.

Six of the diseased animals escaped from their enclosure on the final day of the study and had to be recovered from the veldt where they could not be weighed. Only 11/17 body mass values were available to determine mean body mass for the diseased group of animals. At a 95% confidence level no differences could be detected between the average daily gains of animals with tuberculous lesions compared to those without. There was also no difference between the average

daily gains of the control and vaccine groups. LM 32/z was the only animal with a negative average daily gain and also the most severely affected animal in the trial and had generalized tuberculosis. This animal lost 28kg during the last 2 months of the experiment, her hair was brittle and she was depressed.

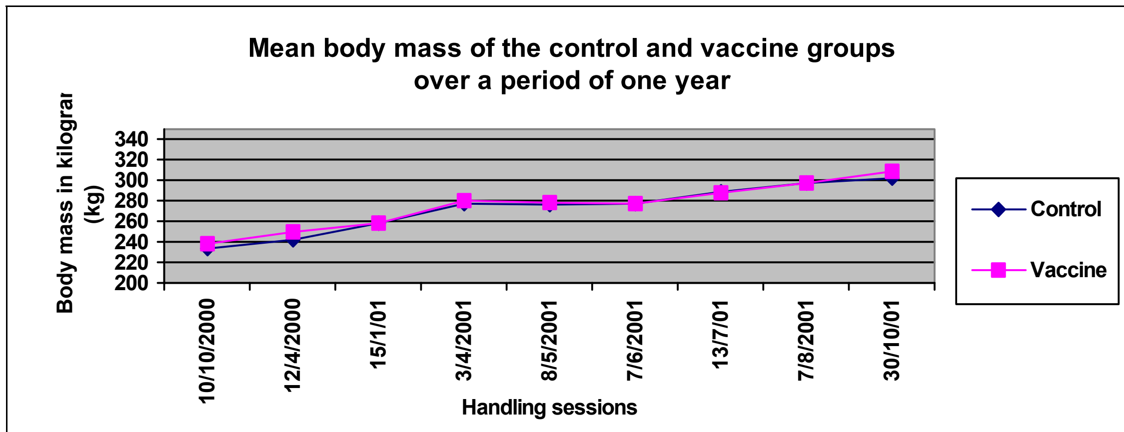

Fig 4.2.1.1: The mean body mass of control and vaccinated animals as measured throughout the duration of the BCG vaccine study.

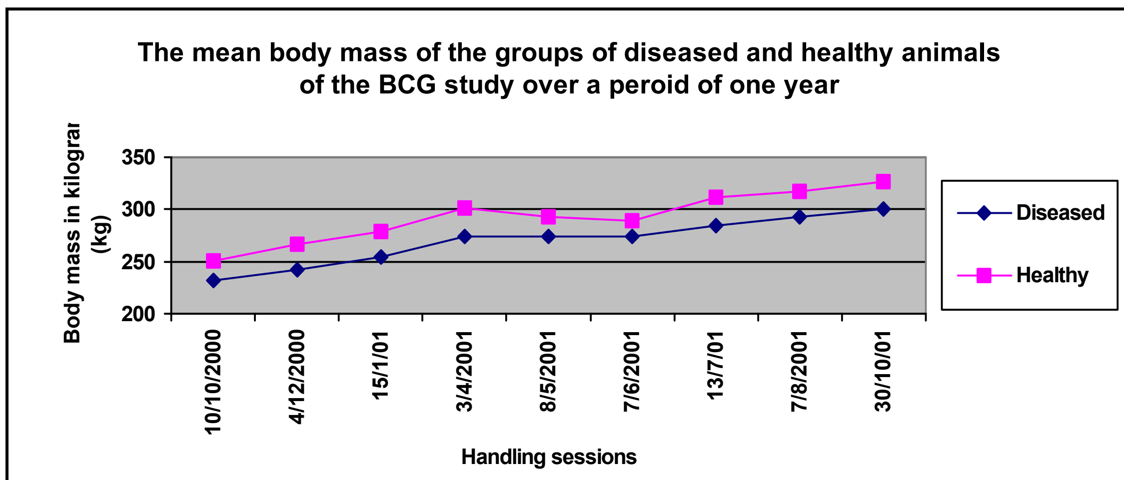

Fig 4.2.1.2: The mean body mass of the groups of diseased and healthy animals as measured during the BCG vaccine study.

4.2.2 Euthanasia

In the BCG vaccine study group, LM 26/z was a smaller animal than the others. He did not adapt well to the boma and was therefore euthanased. This left the control group with 14 animals compared to the 15 animals from the vaccine group.

4.2.3 Comparative intradermal tuberculin test

All the calves selected for the BCG study had negative test results with the first comparative intradermal tuberculin test that was carried out three months prior to the day of intratonsilar challenge. After the intratonsilar inoculations the majority of the calves tested positive with the intradermal tuberculin test. The second intradermal tuberculin test carried out 18 weeks post infection yielded 24 positive test results and 5 negative results. The results for the intradermal tuberculin tests that were performed 32 weeks after *M. bovis* challenge also yielded a total of 24 positive and 5 negative results. However, some of the negative results of the third test were obtained from buffaloes that previously tested positive on the comparative intradermal tuberculin test.

Several of the injection sites in the BCG study showed an immeasurable increase in skin thickness. Immeasurable reactions were mostly associated with peripheral hyperaemia and central foci of necrosis, while some of the other positive reactions only showed mild to moderate oedema. Pain on palpation of the injection sites was evident even though the animals were immobilized and a sero-fibrinous exudate was present, especially when the swollen areas were slightly squeezed. Some animals showed mild oedema at both the avian and bovine PPD injection sites; such responses were regarded as negative.

Table 4.2.3: The results of the second and third intradermal tuberculin tests and a description of the test site reactions observed during the BCG study.

Number	1st ITT	Test site reaction	2nd ITT	Test site reaction	3rd ITT	Test site reaction	*M. bovis* culture
Control							
LM 2/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 4/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 5/z	-	None	+	5,3mm, O	-	None	+
LM 7/z	-	None	+	>33mm, O, H, N	+	13mm, O	+
LM 8/z	-	None	+	17mm, O	+	8,2mm, O	+
LM 9/z	-	None	+	>33mm, O, H, N	+	12,9mm O	+
LM 11/z	-	None	-	None	-	M	-
LM 12/z	-	None	+	5mm, O	+	5,7mm, O	+
LM 15/z	-	None	+	2mm, M	-	Avian reactor, M	-
LM 20/z	-	None	+	>33mm, O, H, N	+	8,8mm, O	+
LM 24/z	-	None	+	10,3mm, O, H, N	+	4mm, O	+
LM 28/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 31/z	-	None	+	>33mm, O, H, N	+	14,8mm, O, H, N	+
LM 33/z	-	None	+	8,5mm, O	+	>33mm, O, H, N	+
Vaccine							
LM 1/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 3/z	-	None	+	7mm, O	+	9mm, O, H, N	-
LM 10/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 14/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 16/z	-	None	+	2mm, M	+	2mm, M, H	+
LM 18/z	-	None	-	M	+	2,4mm, M	-
LM 19/z	-	None	+	>33mm, O, H, N	+	6,8mm, O	+
LM 21/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 23/z	-	None	+	4,8mm, O	+	2mm, M	-
LM 25/z	-	None	-	M	+	3mm, M	+
LM 27/z	-	None	-	M	-	M	+
LM 29/z	-	None	+	>33mm, O, H, N	+	>33mm, O, H, N	+
LM 30/z	-	None	+	5mm, O	+	9mm, O	+
LM 32/z	-	None	+	8,3mm, O	+	8,4mm, O	+
LM 35/z	-	None	-	Avian reactor, M	-	None	+

1st ITT: Comparative intradermal tuberculin test done 12 weeks prior to intratonsilar challenge.

2nd ITT: Comparative Intradermal tuberculin test done at 18 weeks post intratonsilar challenge.

3rd ITT: Comparative Intradermal tuberculin test done at 32 weeks post intratonsilar challenge.

O: Oedema

H: Hyperaemia

N: Necrosis

M: Mild oedema

4.2.4 Necropsy results

Ten of fourteen control animals showed macroscopic evidence of tuberculosis, while 7/15 vaccinated animals had macroscopic lesions. When the affected tissues were evaluated by a pathologist, the macroscopic lesions seemed static in 1/10 of the diseased animals from the control group and 3/7 of the diseased animals from the vaccine group. Tuberculous lesions within the left retropharyngeal lymph nodes varied from pinpoint foci to total replacement of the lymphoid tissue. Although the total number of lymph nodes affected in animals from the control group (26) was higher than that of the vaccine group (15), it was not statistically significant.

LM 32/z (Vaccine group) was the most severely affected animal in this study. The carcass was cachectic and generalized tuberculosis with caseous lymphadenitis in the retropharyngeal, bronchial, mediastinal, hepatic and mesenteric lymph nodes was seen. In some of the lymph nodes the normal structure of the entire lymph node was replaced by a diffuse granulomatous reaction. Tuberculous granulomas varying in size from 5mm to 40mm were seen throughout the lungs and a locally extensive tuberculous pleuritis was present in the left and right thoracic cavity.

The pulmonary lesions that were observed in animals from the BCG study were similar to that seen in wild buffaloes where aerosol infection with live *M. bovis* occurred. Pulmonary lesions were only reported in animals with high lesion scores and ranged from multiple, rather large granulomas in the dorso-caudal lung lobes to miliary caseo-granulomatous foci.

The majority of lymph nodes collected from the animals with higher lesion scores (4 – 5), seemed indurated on palpation, proved difficult to slice and showed a florid granulomatous response. Lesions (pulmonary and lymphoid) from these animals were generally not well encapsulated and moderate to extensive calcification was reported. However, one animal (LM 14/z) from the vaccine group presented with unusual abscessation of the head lymph nodes. Both the retropharyngeal lymph nodes appeared congested, irregularly enlarged and had cavities that were filled

with yellowish-green semi-fluid exudate. These cavities had formed open tracts and the exudate seemed to have drained into the surrounding tissue and the caudal part of the pharynx. Thick (1 – 2mm) capsules were present in both lymph nodes, and the liquid content differed from all lesions seen in others animals from the same study.

With a moderate tuberculous lymphadenitis (score 3) the affected lymph nodes once again seemed indurated, but a difference in size between left and right retropharyngeal lymph nodes was not always reported. Multifocal, calcified, inspisated necrotic lesions were usually observed in these animals and encapsulation could be present or absent. Lower lesion scores (1 – 2) were given to animals where only one lymph node or tonsil had a macroscopic lesion. Lesions within the lymph node were usually small (<5mm diameter) and sometimes only a focal, well-demarcated area of necrosis was observed. Calcification and encapsulation were generally more outspoken in animals with lower lesion scores. These lesions were fibrosed, encapsulated and mineralized and therefore the BTB status was said to be static. In twelve animals no evidence of bovine tuberculosis could be found macroscopically.

Table 4.2.4.1: The number of animals with macroscopic tuberculous lesions in the different sets of specimens collected.

	The number of animals with macroscopic tuberculous lesions in the various lymph node sets					
Group	**Tonsils**	**Head Lnn.**	**Thorax Lnn.**	**Lung**	**Other Lnn.**	**Total number of affected pooled lymph node sets**
Control n = 14	6	10	5	3	2	26
Vaccine n = 15	2	7	3	2	1	15

Although the mean lesion score for animals from the vaccine group was lower (1,5) than animals from the control group (2,5), these results were not statistically significant.

Table 4.2.4.2: The presence of tuberculous lesions in the different lymph nodes, the lesion score, and the disease status of the experimental animals from the BCG study.

Number	Tonsils	Head Inn.	Thoracic Inn.	Other Inn.	Lung	Grading	Disease status
Control							
LM 2/z		X	X		X	5	**Active disease**
LM 4/z		X	X			3	**Active disease**
LM 5/z						0	No visible lesions
LM 7/z	X	X	X	X		5	**Active disease**
LM 8/z	X	X				3	**Active disease**
LM 9/z	X	X				3	**Active disease**
LM 11/z						0	No visible lesions
LM 12/z		X				1	**Active disease**
LM 15/z						0	No visible lesions
LM 20/z		X				2	Static disease
LM 24/z						0	No visible lesions
LM 28/z	X	X				3	**Active disease**
LM 31/z	X	X	X		X	4	**Active disease**
LM 33/z	X	X	X	X	X	5	**Active disease**
Vaccine							
LM 1/z		X				2	Static disease
LM 3/z						0	No visible lesions
LM 10/z	X	X	X			3	**Active disease**
LM 14/z	X	X		X		4	**Active disease**
LM 16/z						0	No visible lesions
LM 18/z						0	No visible lesions
LM 19/z		X	X		X	5	**Active disease**
LM 21/z		X				1	Static disease
LM 23/z						0	No visible lesions
LM 25/z						0	No visible lesions
LM 27/z						0	No visible lesions
LM 29/z		X				1	Static disease
LM 30/z						0	No visible lesions
LM 32/z		X	X	X	X	5	**Active disease**
LM 35/z						0	No visible lesions

Figures 4.2.4.1 – 4.2.4.6 indicate the range of lesions that were observed in animals from the BCG study.

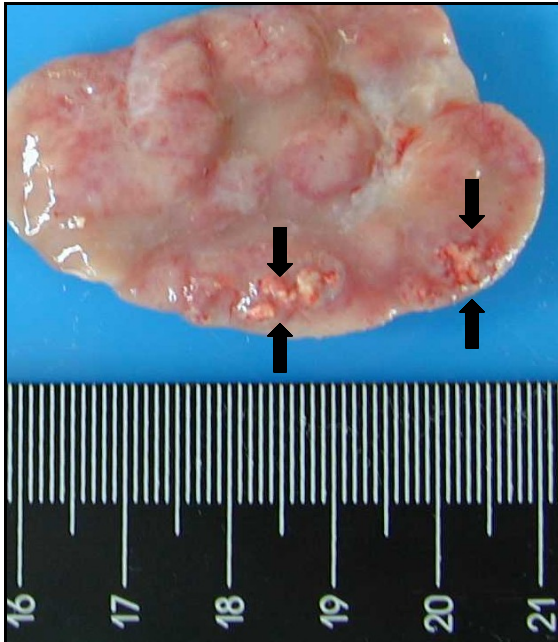

Fig 4.2.4.1: Small, multifocal, well demarcated lesions in the retro-pharyngeal lymph node usually seen in animals with lower lesion scores.

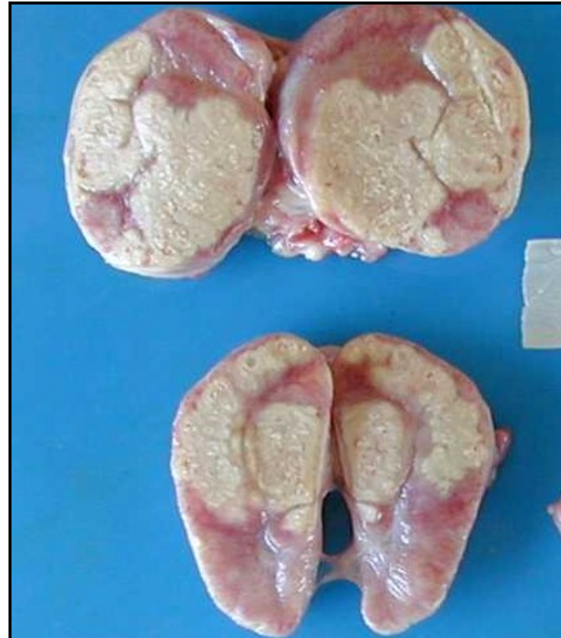

Fig 4.2.4.2: Severe lymphadenitis with necrosis and calcification. More than 50% of the normal lymph node structure has been replaced.

Fig 4.2.4.3: The normal structure of the left retropharyngeal lymph node has been replaced by a caseous necro-granulomatous reaction.

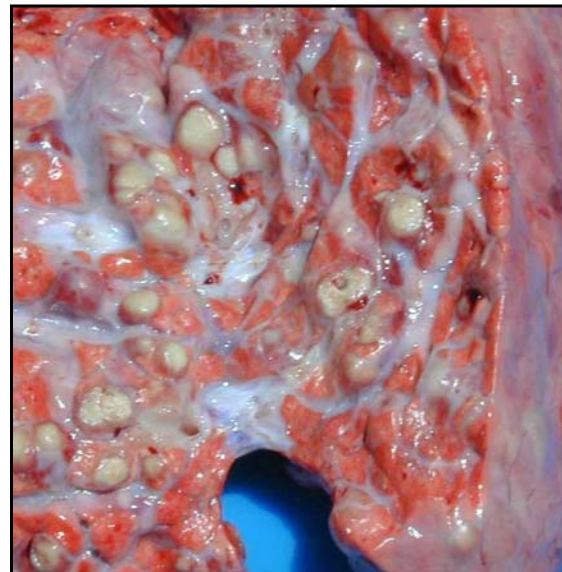

Fig 4.2.4.4: Miliary lesions through-out the lung parenchyma.

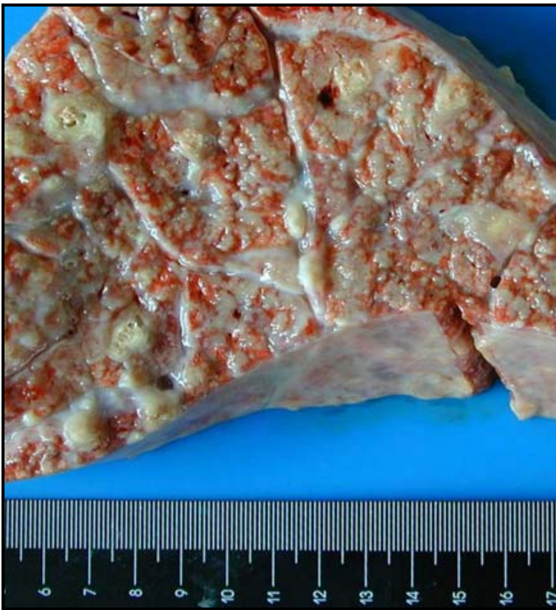

Fig 4.2.4.5: Multifocal to confluent pyogranulomatous pneumonia.

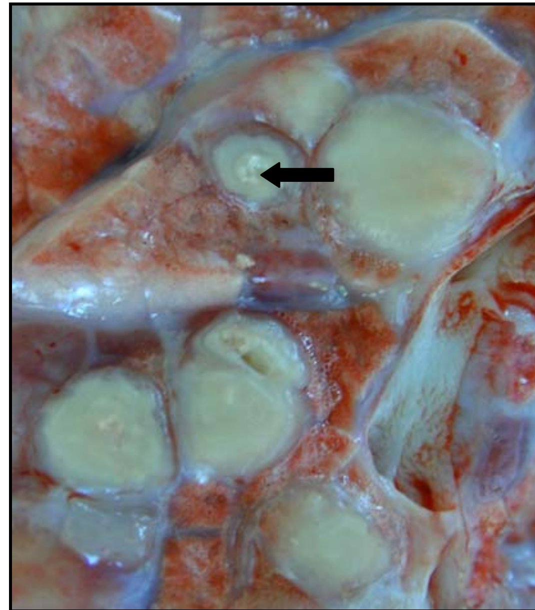

Fig 4.2.4.6: Caseo-necrotic granulomas with some calcification in the lung parenchyma.

4.2.5 Laboratory tests

4.2.5.1 Haematology

A marked decrease in lymphocyte counts occurred from 9 weeks to 18 weeks post infection. In some animals the lymphocyte counts were down to one third of the normal value for the individual animal, but in the majority of cases there was a 40 – 50% decrease in the lymphocyte counts. The lymphopenia coincided with the foot-and-mouth disease outbreak. The lymphocyte counts returned to normal two to three months after the foot-and-mouth disease outbreak.

The only animal that showed changes in haematological parameters towards the end of the study was LM 32/z. A decrease in red blood cell count was noted, with values ranging between 5.13×10^6 – 7.34×10^6 where normal values usually range between 7×10^6 – 12×10^6 (ISIS Clinical Pathology Values 1999). There was also a decline in haematocrit values ranging from 21% – 26.3% (normal value:

29% - 51%) as well as a decrease in haemoglobin concentration with values between 7.5g/dl – 9.9g/dl (Normal: 10.5g/dl – 16.5g/dl). For the rest of the animals no difference could be detected between the haematological values of diseased and healthy animals at a confidence level of 95%.

Individual haematology results of all the animals from the BCG vaccine study are available in Annexure II, Tables 5.1 & 5.2. A summary of the mean haematological values of diseased and healthy animals can be seen in Annexure II, Table 5.3.

4.2.5.2 Interferon-gamma (IFN-γ) assay

The blood of all experimental animals was subjected to two IFN-γ assays prior to the date of primary vaccination. The first test was carried out just after capture and the next test two weeks prior to vaccination. The IFN-γ assay results were negative for all experimental animals before the start of the BCG vaccine trial.

The IFN-γ assays were repeated throughout the study at regular intervals. Results from IFN-γ assays carried out at 6 weeks prior to intratonsilar infection, on the day of intratonsilar infection and at four, nine, thirteen, eighteen, twenty-two, twenty-six, thirty-one and thirty-four weeks post infection are summarized in Table 4.2.4.2. After conversion to a positive IFN-γ result, the majority of animals continued testing positive. However, some differences in the IFN-γ results were recorded from four buffalo calves, two each from the control and vaccinated groups, respectively. These calves reacted unusual by reverting to negative results on two or more consecutive tests, after previously testing positive.

The final results of the IFN-γ assay were correlated with *M. bovis* culture results as well as the presence of macroscopic lesions. Macroscopic lesion results were as follows: 10/14 control animals had lesions and 4/14 were negative, while 7/15 vaccinated animals had lesions and 8/15 were negative.

Table 4.2.5.2: Results of the IFN-γ assay at regular intervals throughout the BCG vaccine study

Number	2nd V	Day 0	4wks	9wks	13wks	18wks	22wks	26wks	31wks	34wks	Result
Control											
LM 2/z	-	-	+	-	+	+	+	+	+	+	Positive
LM 4/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 5/z	-	-	-	-	+	-	-	-	-	-	Negative
LM 7/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 8/z	-	-	+	-	+	+	+	+	+	+	Positive
LM 9/z	-	+	-	+	+	+	+	+	+	+	Positive
LM 11/z	-	-	-	-	-	-	-	-	-	-	Negative
LM 12/z	-	-	-	+	+	-	+	+	-	+	Positive
LM 15/z	-	+	-	-	-	-	-	-	-	-	Negative
LM 20/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 24/z	-	-	-	+	+	+	+	-	-	+	Positive
LM 28/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 31/z	-	-	+	+	+	+	+	+	+	+	Positive
LM 33/z	-	-	+	-	+	+	+	+	-	+	Positive
Vaccine											
LM 1/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 3/z	-	-	-	-	+	-	-	-	-	-	Negative
LM 10/z	-	+	+	+	+	+	+	+	+	+	Positive
LM 14/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 16/z	-	-	+	-	-	+	+	-	-	+	Positive
LM 18/z	-	-	-	-	-	-	-	-	-	-	Negative
LM 19/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 21/z	-	+	-	+	+	+	+	+	+	+	Positive
LM 23/z	-	-	-	-	-	-	-	-	-	-	Negative
LM 25/z	-	-	-	-	+	-	-	+	-	-	Positive
LM 27/z	-	-	-	-	-	-	-	-	-	-	Negative
LM 29/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 30/z	-	+	-	+	-	-	+	-	-	+	Positive
LM 32/z	-	-	-	+	+	+	+	+	+	+	Positive
LM 35/z	-	-	-	-	-	-	-	-	-	-	Negative

2nd V: Day on which secondary vaccinations were given (6 weeks prior to intratonsilar infection)

Day 0: The day of intratonsilar infection

wks: The number of weeks post intratonsilar infection that the γ-IFN assays were carried out.

4.2.5.3 Histopathology

Calcification and necrosis were more pronounced in the control animals and more active lesions were reported on histopathology. Encapsulation of lesions was better developed in the vaccinated animals than in animals from the control group. The histopathology results confirmed that most of the lesions seen macroscopically, were TB granulomas. Two animals without any visible lesions had positive histopathology results (lesions could have been too small to see with the naked eye), while in one animal (LM 12/z), the lesion seen macroscopically could not be detected histologically.

4.2.5.4 Acid-fast organism (AFO) count

Acid-fast organisms were observed in only 5/10 control animals with positive histopathology results. In 3/4 of the above cases (LM 4/z, LM 7/z & LM 20/z) fewer than 5 AFOs were seen per histopathological section. One animal (LM 33/z) had 10 – 20 AFOs per section and in the last case (LM 2/z), numerous AFOs were encountered per section. AFOs could only be seen in 3/8 animals from the vaccine group that had a confirmed TB-positive status on histopathology. LM 30/z had < 5 AFOs per microscopic field, LM 19/z had 10 – 20 AFOs per field and LM 32/z had numerous AFOs on four different histopathology sections. None of the acid-fast organism count results were statistically significant.

Table 4.2.5.3: The histopathology results and the acid-fast organism counts of the lymph nodes removed from the experimental buffalo calves from the BCG study at necropsy.

Number	Lymph node	AFO count per slide	AFO score	Histopathology result
Control				
LM 2/z	L. retropharyngeal	Multiple	4	Positive, active[1]
	Bronchial	Multiple	4	
	Mediastinal	Multiple	4	
LM 4/z	L. retropharyngeal	< 5	1	Positive, active
LM 5/z	L. retropharyngeal	None	0	Negative
LM 7/z	L. retropharyngeal	< 5	1	Positive, active
	Bronchial	5 – 10	2	
LM 8/z	L. retropharyngeal	None	0	Positive, active
LM 9/z	L. retropharyngeal	None	0	Positive, static[2]
LM 11/z	L. retropharyngeal	None	0	Negative
LM 12/z	L. retropharyngeal	None	0	Negative
LM 15/z	L. retropharyngeal	None	0	Negative
LM 20/z	L. retropharyngeal	< 5	1	Positive, active
LM 24/z	L. retropharyngeal	None	0	Positive, static
LM 28/z	L. retropharyngeal	None	0	Positive, active
LM 31/z	L. retropharyngeal	None	0	Positive, active
	Mediastinal	< 5	1	
LM 33/z	L. retropharyngeal	10 – 20	3	Positive, active
	Bronchial	10 – 20	3	
Vaccine				
LM 1/z	L. retropharyngeal	None	0	Positive, static
LM 3/z	L. retropharyngeal	None	0	Negative
LM 10/z	L. retropharyngeal	None	0	Positive, active
LM 14/z	L. retropharyngeal	None	0	Positive, active
LM 16/z	L. retropharyngeal	None	0	Negative
LM 18/z	L. retropharyngeal	None	0	Negative
LM 19/z	L. retropharyngeal	10 - 20	3	Positive, active
	Bronchial	10 - 20	3	
	Mediastinal	< 5	1	
LM 21/z	L. retropharyngeal	None	0	Positive, static
LM 23/z	L. retropharyngeal	None	0	Negative
LM 25/z	L. retropharyngeal	None	0	Negative
LM 27/z	L. retropharyngeal	None	0	Negative
LM 29/z	L. retropharyngeal	None	0	Positive, static
LM 30/z	L. retropharyngeal	< 5	1	Positive, static
LM 32/z	L. retropharyngeal	Multiple	4	Positive, active
	Bronchial	Multiple	4	
	Mediastinal	Multiple	4	
	Mesenteric lnn.	Multiple	4	
LM 35/z	L. retropharyngeal	None	0	Negative

[1 & 2] **Static** or **active:** Referring to the type of microscopic lesion observed.

4.2.5.5 *Mycobacterium bovis* culture

Only two nasal swabs yielded positive culture results for *M. bovis* during the trial period of the BCG vaccine study. These swabs were taken with a one month interval from the same animal (LM 10/z). The majority of the swabs yielded no mycobacteria, but contaminated cultures were often seen.

Table 4.2.5.5: The number of animals with positive *M. bovis* culture results in the different pooled samples of lymph nodes and tissue specimens.

	Left tonsil	**Right tonsil**	**Left Retro. ln.**	**Right Retro. ln.**	**Head Pool Lnn.**	**Thorax Pool Lnn.**	**Lung**	**Abdom. Pool Lnn.**	**Carcass Pool Lnn.**
Control Group n = 14	7	1	10	1	1	5	3	1	1
Vaccine Group n = 15	7	1	8	2	1	5	2	1	1

Twelve of fourteen control animals had positive cultures while 12/15 vaccinated animals also cultured positive for *M. bovis*; thus a total of 24 positive and 5 negative. There was no difference between the BTB culture results of control versus vaccinated animals. The mycobacterial culture results were as expected, with the majority of left retropharyngeal lymph node specimens (18/29) culturing positive for *M. bovis*. *Mycobacterium bovis* was cultured from 14/29 left tonsil pools, while the thoracic lymph node pools gave 10/29 positive cultures.

Twelve weeks was the cut-off time for retrieval of *M. bovis* isolates. If no growth occurred within the first 12 weeks after incubation, the result was regarded as negative.

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4.2.6 Summary of the results from the BCG vaccine study

Table 4.2.6: Summary of the different field and laboratory tests.

Nr.	Gender	Skin test	IFN-γ	Macroscopic lesion	Histopathology	AFO counts	*M. bovis* culture
Control group							
LM 2/z	M	+	+	+	+	Multiple	+
LM 4/z	M	+	+	+	+	< 5	+
LM 5/z	F	-	-	-	-	None	+
LM 7/z	F	+	+	+	+	< 5	+
LM 8/z	F	+	+	+	+	None	+
LM 9/z	F	+	+	+	+	None	+
LM 11/z	F	-	-	-	-	None	-
LM 12/z	F	+	+	+	-	None	+
LM 15/z	F	-	-	-	-	None	-
LM 20/z	F	+	+	+	+	< 5	+
LM 24/z	M	+	+	-	+	None	+
LM 28/z	F	+	+	+	+	None	+
LM 31/z	M	+	+	+	+	< 5	+
LM 33/z	M	+	+	+	+	10 – 20	+
Vaccine group							
LM 1/z	M	+	+	+	+	None	+
LM 3/z	F	+	-	-	-	None	-
LM 10/z	M	+	+	+	+	None	+
LM 14/z	F	+	+	+	+	None	+
LM 16/z	F	+	-	-	-	None	+
LM 18/z	M	+	-	-	-	None	-
LM 19/z	F	+	+	+	+	10 – 20	+
LM 21/z	M	+	+	+	+	None	+
LM 23/z	F	+	-	-	-	None	-
LM 25/z	F	+	+	-	-	None	+
LM 27/z	F	-	-	-	-	None	+
LM 29/z	F	+	+	+	+	None	+
LM 30/z	M	+	+	-	+	< 5	+
LM 32/z	F	+	+	+	+	Multiple	+
LM 35/z	F	-	-	-	-	None	+

CHAPTER V

5 CONCLUSION AND DISCUSSION

5.1 *CONCLUSION*

5.1.1 Infection Model

The infection model produced lesions comparable to natural infection in the African buffalo. The laboratory tests were able to differentiate from an early stage between infected and healthy buffalo calves. Depending on the infective dose, animals infected with *M. bovis* can be expected to develop macroscopical lesions as early as 5 weeks after exposure. The rate of development of the lesions and the extent of lesions were dose related, because more animals from the high dose group had macroscopic lesions and the granulomatous reactions involved a greater percentage of the lymph node mass. The high dose (HD) of 3×10^4 cfu of live *M. bovis* was considered the dose of choice for future trials since it affected more animals.

5.1.2 BCG Vaccine study

Under the prevailing conditions the BCG-Pasteur (1173P2) vaccine was not able to protect the buffalo calves against *Mycobacterium bovis* infection. Although fewer vaccinated animals developed macroscopic lesions, the differences from the control group were not statistically significant. The laboratory tests differentiated between infected and healthy animals. Clinical foot-and-mouth disease caused a greater loss in body mass over a short period of time than did BTB. Weight loss, hair loss, coughing and general weakness only manifested when advanced tuberculosis was present. Haematological parameters are not good indicators of the TB status of buffaloes, except where the disease has progressed to its end stage. A decline in the haematocrit and haemoglobin concentration parameters was then seen. Mycobacterial culture results are critical for confirmation of

infection, because some animals still yielded positive culture results after detailed necropsy procedures were followed and no tuberculous lesions could be seen even at 34 weeks post infection. This might indicate the ability of *M. bovis* to survive within the lymphoid tissue for very long periods at a time.

5.2 *DISCUSSION*

As was expected from a live *M. bovis* intratonsilar challenge, all diseased animals had tuberculous lesions in the left retropharyngeal lymph node because this lymph node drains the palatine tonsil. All the tuberculous lesions observed showed caseous necrosis as well as central calcification, and were generally indistinguishable from lesions usually seen in naturally-infected buffaloes. Not all infected animals developed lesions, and this was true especially in the group of animals challenged with the low dose. Only 2 animals developed secondary BTB lesions, one each from the LD and HD groups, respectively.

In a study done by Palmer *et al* (1999), where cattle were also inoculated with live *M. bovis* via the tonsil, all cows had developed granulomas in the medial retropharyngeal lymph nodes by 6 weeks post infection. At 8 weeks post infection granuloma development was more advanced and lesions were also observed in other lymph nodes, indicating that mycobacterial spread to distant sites most likely occurred through the lymphatic route (Whipple, Bolin & Miller 1996). Similar findings were also reported by Mackintosh *et al*, where red deer were challenged with 100 – 500 cfu of live *M. bovis* via the tonsilar route. Tuberculous lesions were primarily reported in the medial retropharyngeal lymph nodes and the spectrum of pathological and immunological responses were typical of naturally occurring BTB in deer.

BTB lesions were found in the left retropharyngeal lymph nodes of the majority of diseased calves, but there was quite a variation in the severity of the lesions. The severity of the lesions was found to be related to the challenge dose of *M. bovis*. The macroscopic lesions seen in the HD group were more extensive than those

seen in LD animals and the number of diseased animals was also higher in the HD group. No animals from the Infection Model developed pulmonary disease as reported by Cassidy *et al* (1998), when the latter infected domestic calves intra-nasally. The severity of pulmonary disease seen in their animals appeared to be related to the dose (10^7 cfu) of live *M. bovis* administered, which was higher than the challenge of our HD group. Another important factor is the time frame of the Infection Model. The calves were slaughtered at 22 weeks post infection and such a short period may not have been enough for spread of disease to distant sites from the initial entry site of infection.

The tuberculous lesions reported in diseased animals from the BCG vaccine study were much more extensive than lesions seen in animals from the Infection Model study. The main reason for this could be the duration of the study, but the live *M. bovis* challenge dose, stress and competition for feeding space might also have played a role. The calves from the BCG study were euthanased at 34 weeks post infection, thus being infected for 12 weeks longer than calves in the Infection Model experiment. In the case of the BCG study, the animals had a longer period of time to develop severe tuberculous lesions. The left retropharyngeal lymph node was once again more often involved in lesion development than any of the other lymph nodes. In the BCG study, however, several animals were reported to have tonsilar, thoracic lymph node and lung involvement as well. Three cases of generalized tuberculosis were reported (LM 2/z, LM 31/z, LM 32/z), and a total of five animals received the highest obtainable lesion score of 5.

Surprisingly, a vaccinated animal was the worst BTB case found at the end of the study, not only showing generalized TB with a severe tuberculous pneumonia and pleuritis, but also clinical manifestations of hair loss, weight loss and malaise. Although there were fewer vaccinated animals (7/15) with macroscopic lesions than control animals (10/14), this observation was not statistically significant ($p > 0.05$). The total lesion score was also lower in the vaccine group than in control animals, but this was expected as fewer diseased animals were present in the

vaccine group. It was also found that lesions from vaccinated animals were generally well encapsulated and static when compared to those in control animals, and might be an indication that they were less likely to shed live *M. bovis.* However, this statement could not be statistically justified.

Corner *et al* (2002) reported similar results, where possums that received multiple vaccinations showed less pulmonary lesions and a lower lung weight compared to control animals or animals that received a single vaccination. If vaccination of animals can lead to a lesser degree of disease there may be merit in the vaccination of wildlife populations, because the risk of animals becoming shedders of live *M. bovis* can be diminished. This scenario is also true for human medicine where the BCG vaccine is believed to influence the severity of disease but not necessarily protect against infection. It was also pointed out by the Centers for Disease Control and Prevention (CDC) in the USA, that although the BCG vaccine does not protect against infection in the long term, it definitely limits development of pulmonary tuberculosis and serious disease in children (Simpson 2003; CDC 1996).

The buffalo calves did not show any loss in body mass throughout the duration of the Infection Model study. In buffaloes, BTB usually has a subclinical development followed by clinical manifestations such as poor body condition and weight loss as chronicity progresses (Bengis *et al* 1996; De Vos 2001). One would have expected the animals with more severe tuberculous lesions from the HD group to experience some loss in body mass and condition, but the *M. bovis* infection did not seem to have any effect on the bodily conditions or gaining of body mass of these young buffaloes during the first 6 months post infection. It is likely that the 6-month duration of the study was too short for a disease with a chronic development to have progressed to the extent that it could affect their general health. However, the decline in average daily gain of the LD group was due to the fact that both animals that lost weight during the last month of the trial period belonged to this group. Both cases of weight loss were unrelated to bovine tuberculosis. One animal had a focal

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peritonitis due to a puncture wound in the reticulum because of a dart that was swallowed, and the other showed no specific indication as to what could have caused the weight loss.

Similar results were seen in the BCG vaccine study where no statistically significant difference could be detected between the average daily gains of diseased and healthy animals or between animals from different treatment groups. However, some loss in body mass was reported in the majority of animals from the BCG study from 9 to 18 weeks post intratonsilar challenge, which coincided with a clinical foot-and-mouth (FMD) outbreak. The larger animals were more adversely affected with a greater loss in body mass than the younger and smaller buffalo calves. When the loss of body mass and condition during the FMD outbreak was compared to the TB status of the animals, it could clearly be seen that the virus had a more severe influence on body mass and condition than bovine tuberculosis had at that stage. Severe oral lesions were found on the dorsal and lateral tongue surfaces as well as the dental pads in the majority of experimental animals. These lesions caused pain and discomfort, therefore the animals soaked their mouths in the drinking troughs for long periods at a time. The oral cavities were too sensitive for the animals to eat normally. The inability to feed due to a painful oral cavity was probably the most important factor in causing weight loss in this group of animals. Only one animal showed weight loss towards the end of the study, losing 28kg in body mass over a period of 2 months. This was the most severely affected experimental animal with advanced generalized tuberculosis and therefore it is believed that the cause of weight loss in this animal was TB related.

It is believed that an FMD outbreak could have occurred as a result of excessive stress on the buffalo calves. Young, Hedger and Howell reported a similar clinical outbreak of FMD in young buffalo held in captivity in the KNP in 1972. However, the FMD outbreak was not the only stress factor present during the BCG vaccine study. When diarrhoea was observed, the intestinal parasite loads of the animals were determined to decide on treatment. Coccidia outbreaks occurred during both

the studies, but were noticeably more severe during the vaccine study due to the increased number of animals present in the enclosures. Blanket treatment with Amprolium 30% (added to the drinking water) was usually successful in terminating such an outbreak.

The lymphocyte function of animals might also be suppressed due to causes such as viral infections, stress or metabolic disease (Lawler Goff 1996). A decline in lymphocyte count also coincided with the FMD outbreak. Lymphocyte counts were generally 40% - 50% lower during the outbreak than what was observed 4 weeks prior to or 8 weeks after the FMD outbreak. All the animals from the BCG Vaccine study tested positive on the ELISA, indicating that serum antibody against the disease was present in all the animals. As previously discussed, antibody production is regulated by IL-4 which is produced by the T_H2 response. In the light of the fact that very high levels of circulating antibody against the FMD virus was present and that T_H1 and T_H2 responses operate in reciprocal fashion, it is therefore believed that a humoral rather than cellular immune response was stimulated. For successful vaccination of animals against bovine tuberculosis, a proper T_H1 response is necessary in order for the macrophages to rid the host of the mycobacteria. The presence of the FMD virus caused a bias in the immune response from T_H1 to T_H2. Therefore, the cellular immune response was not optimal, and the value of the animals as candidates for a vaccination trial diminished.

Evaluation of the haematology profiles did not indicate any differences between diseased and healthy animals form either of the experimental studies at a confidence level of 95%. The changes in haematology parameters from only one animal in the BCG vaccine study could be ascribed to the fact that the disease was already in an advanced stage and the animal was also manifesting clinical signs such as emaciation, hair loss and depression. These changes in haematological parameters included a decrease in red blood cell count, a drop in haematocrit as well as a decrease in haemoglobin concentration. As these changes were only

observed in the single animal with clinical presentation of tuberculosis it is suggested that haematological parameters are indicative only of advanced disease and not of BTB infection. The only other notable observation was the decline in lymphocyte counts during the FMD outbreak in the BCG vaccine study.

In the study of Kumar & Parihar (1998) where water buffalo calves (n = 6) were infected intravenously (IV) and bulls (n = 6) intratracheally (IT) with live *M. bovis* some significant changes in the haematological parameters were noted. The total erythrocyte count (TEC), haematocrit (Ht) and haemoglobin (Hb) values were significantly decreased in that group of calves. All the calves died within the first 60 days of the experiment indicating that the intravenous infection has caused an overwhelming generalized disease that ultimately lead to the death of the animals. An intravenous infection is not a natural way of disease transmission and one should thus be careful to compare these results. The adult bulls from the IT group did not have any of the above-mentioned changes but a significant decrease in total serum proteins occurred from 270 days post infection, which could be ascribed to general debilitation after the onset of disease. A significant decrease in neutrophil counts was observed in these animals during the later stages of the disease. These observations could not be confirmed in the Infection Model study.

The comparative intradermal tuberculin test is considered to be a reliable test in the buffalo and therefore used as a prerequisite for the testing of disease-free buffaloes, but it has a lower specificity than the gamma-interferon test. Two other problems associated with the intradermal tuberculin test, are the interval of testing and the cost factor. The animals have to be immobilised twice therefore making the test expensive and the test can only be done once every 3 – 4 months. The sensitivity of the intradermal tuberculin test using bovine PPD in buffalo calves however, compared well with that of the IFN-γ assay. More important however, is the fact that the IFN-γ assay can be run at very short intervals which is not possible with the intradermal tuberculin test. The IFN-γ assay was also able to distinguish

from as early as 4 weeks post intratonsilar challenge between diseased and healthy animals.

Two animals from the control group in the BCG Vaccine study had negative results on their third intradermal tuberculin tests compared to previous positive results. None had macroscopic lesions and one was positive and one negative on *M. bovis* culture. It can be speculated that animals with dormant infections fail to respond to PPD stimulation or that repeated testing of animals with PPD increase the number of animals failing to respond (Thoen & Bloom 1995). However, there is also the possibility of a false positive reaction, especially in the animal with a negative *M. bovis* culture result. Francis, Seiler, Wilkie, O'Boyle, Lumsden and Frost (1978) reported on the sensitivity and specificity of the various tuberculin tests using bovine PPD and other tuberculins. They concluded that the intradermal tuberculin test on the side of the neck delivered the highest possible sensitivity while the caudal tail fold test showed the highest possible specificity. However, they found the comparative intradermal tuberculin test to be less sensitive and specific and therefore suggested that only single tests should be used for surveys. A different observation applied to the buffaloes when injected with bovine and avian PPDs. The comparative intradermal tuberculin test was more successful in detecting true bovine reactors than when bovine PPD was used alone (pers. comm. Bengis, Skukuza 2001). Due to the presence of other mycobacterial species in the KNP, most buffaloes show some reaction to the intradermal PPD injections. It is therefore important to differentiate between true bovine reactors and animals that react to the avian tuberculin as well. However, the intradermal tuberculin test was still confusing at times due to false positive as well as false negative reactions.

In general, attempts to culture mycobacteria from nasal swabs yielded poor results, and *M. bovis* could only be cultured from two swabs from a single animal. Numerous factors may have influenced the poor results. Environmental contaminants of the nasal mucosae could have inhibited the growth of *M. bovis*, if it in fact had been present in the nasal conchae. If an animal with open tuberculous

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lung lesions did not cough during the few hours preceding specimen collection, it was unlikely that live *M. bovis* would be present in the nasopharynx at that time. The swabs also may have been too short to reach the areas of the deep nasal conchae. Diseased animals usually shed the organism intermittently. Nasal swabs collected for culture are therefore not reliable specimens to determine the TB status of an animal.

M. bovis was cultured from all the macroscopic tuberculous lesions that were submitted for mycobacterial culture from the Infection Model study. Two animals from the LD group and 2 from the HD group with no macroscopic BTB lesions in the retropharyngeal lymph nodes also had positive culture results for *M. bovis*. This could indicate that the live *M. bovis* can be harboured within the lymphoid tissue without the development of caseous necrosis for the first 22 weeks post infection. Whether these animals would have eventually developed BTB lesions is unknown. Although infected and control animals were housed on the same premises, but not in direct contact, none of the control animals ever tested or had positive culture results for *M. bovis*. This confirms the perception that mycobacteriosis is an air-borne disease that can only infect animals within very short distances from one another. Menzies & Neill (2000) stated that *M. bovis* can only survive in the environment for a few weeks at most and that the mycobacterium is very rarely isolated from soil and pasture samples. Cattle-to-cattle transmission through naturally contaminated pasture also failed to cause disease. It can therefore be concluded that direct contact of an infected animal with a healthy animal is needed for the disease to be transmitted, hence the term nose-to-nose disease.

All specimens containing macroscopic lesions submitted for culture from animals in the BCG vaccine study, yielded positive *M. bovis* culture results. The majority of sample sets started showing mycobacterial growth after only 2 – 3 weeks of incubation. However, there were some specimens that needed 6 – 12 weeks of incubation for mycobacterial growth to occur. These specimens were mostly represented by the animals from the vaccine group. The BCG vaccine could have

had some influence on the survival rate of the live *M. bovis*, and therefore very few organisms survived within lymphoid tissue. The low number of organisms present within the lymph node meshwork could explain the long period of time that was needed for mycobacterial growth to be observed. It is therefore suggested that BCG vaccination lowered mycobacterial load in the experimental animals.

The observation that some specimens that yielded positive culture results did not contain macroscopic lesions was quite common among animals from the BCG vaccine study. Even at 34 weeks post infection, some individuals had no signs of macroscopic TB lesions but the retropharyngeal lymph nodes yielded positive cultures. It can thus be speculated that these animals might have had greater genetic resistance against TB or the vaccine may have played some role in stimulating a protective immune response thereby eliminating the live *M. bovis*. Due to the fact that samples were collected from the macropathology seen, it was inevitable that the histopathology results compared better with the necropsy results than those from the mycobacterial cultures. Although 8/15 vaccinated animals and 10/14 control animals had positive histopathology results, the difference was not significant at the 95% confidence level. Eight of ten control animals were histologically classified as having active disease compared to the 4/8 animals from the vaccine group, but these results were still not statistically significant ($p > 0.05$). Only 5 animals from the vaccine study had negative culture results, 2 from the control group and 3 being vaccinated animals. Although some differences were reported in macroscopic TB results and activity of disease between animals from the different treatment groups in the vaccine study, the culture results indicated that there was no difference in the BTB infection status.

When considering infection and disease it is important to understand that all diseased animals are infected, but not all infected animals necessarily become diseased. This mainly refers to the fact that some animals tested positive for *M. bovis* as well as having positive culture results, but no macroscopic signs of tuberculosis were present in the carcasses. The final results of the Infection Model

revealed that the high dose of live *M. bovis* caused 100% infection and 82% disease in that group of experimental animals. In contrast to the results from the high dose group, the low dose caused infection in only 55% of the experimental animals and 36% disease.

In retrospect, the dose of choice might actually have been too high to simulate natural infection in buffalo calves. It may have been better to select a dose that only caused disease in 50% of the animals rather than 82%. When the lesions in the lymph nodes from the animals in the BCG vaccine study were examined, it became clear that the TB lesions were more extensive than expected. In the BCG vaccine study the high dose caused 86% infection and 71% disease, which is lower than what was observed during the Infection Model study, but probably still too high because the spread of disease to secondary sites was recorded in several cases. Apart from embolic spread to secondary sites, pulmonary lesions could also have developed due to inhalation of the live organisms just after instillation into the tonsilar crypt. When such a high challenge dose is used, one might be overriding the protective effect of the vaccine being tested. It is, however, very difficult to decide on the proper challenge dose, because the natural dose of live *M. bovis* within the aerosolized excreta from any diseased buffalo is not known.

Under the prevailing conditions the BCG vaccine was not effective in protecting the buffalo calves against infection and disease. There may be several reasons for vaccine failure, one of which is that the BCG-Pasteur vaccine was unable to elicit a proper immune response against *M. bovis* in the African buffalo. Another possibility could be the presence of extrinsic factors such as stress and viral disease that caused the vaccine to fail. The fact that a lymphopaenia was recorded during the foot-and-mouth outbreak may have caused an inappropriate T cell response without the necessary cellular immunity that is of crucial importance when vaccine efficacy is considered. The challenge dose may have been too high, having an overriding effect on the immune response and thus causing rapid disease progression. A similar dose of BCG-Pasteur vaccine was able to protect red deer

infected with similar live *M. bovis* doses to what was used in this experiment. Although the immune responses in buffalo cannot be compared to those in red deer, it is suggested that the *M. bovis* strain from the KNP might be more virulent than *M. bovis* strains elsewhere in the world. Unfortunately very little is known about the virulence of different *M. bovis* strains throughout the world.

In view of the fact that stress may have influenced vaccine efficacy, it is recommended that the BCG vaccine trial be repeated in a less stressful environment where, for example, outbreaks of coccidiosis and foot-and-mouth disease can be avoided. It has been shown experimentally by Griffin *et al* (1999), that the effect of increased levels corticosteroids totally ablated the efficacy of the BCG vaccine. Studies of resistance heritability in the African buffalo population might also be beneficial. This might give an indication of whether selection for naturally resistant animals could lower the disease prevalence in a free-ranging population.

A second recommendation is that a method of natural horizontal infection should be applied so that the efficacy of the vaccine can be tested against natural infection. However, in such an experiment the date of infection will be unknown as will the infectious dose of live *M. bovis*, but disease progression is believed to be slower. Should natural disease transmission not occur, a lower challenge dose is recommended to be administered via the intratonsilar route. Mackintosh *et al* (1995) proved that as few as 50 colony forming units were able to induce clinical bovine tuberculosis in red deer. If the number of experimental animals can be increased, results that are statistically significant may be obtained. Based on a power calculation done on results of the BCG vaccine study, statistical significant differences could have been obtained if the N-number of both groups was increased to 40 animals per group. However, the importance of N-numbers when working with wild animals remains a sensitive issue. Finally it is recommended that the virulence of the challenge strain be compared to other *M. bovis* isolates.

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Annexure I

Table 1: Infection model animals

Control group (Group 3)		Low dose (Group 2)		High dose (Group 1)	
Animal number	**Sex**	**Animal number**	**Sex**	**Animal number**	**Sex**
LM 4	M	LM 1	M	LM 3	F
LM 5	M	LM 2	M	LM 6	M
LM 8	F	LM 7	F	LM 10	F
LM 9	F	LM 12	**F**	LM 11	F
LM 18	F	LM 14	F	LM 13	F
LM 26	M	LM 17	F	LM 15	M
		LM 21	F	LM 16	M
		LM 22	M	LM 19	F
		LM 23	M	LM 20	M
		LM 24	F	LM 25	M
		LM 27	M	LM 28	M

Table 2.1: Live body mass of animals from the high dose (HD) group

HD group	31/01/00	6/3/00	17/04/00	25/06/00	Total gain	Average daily gain
LM3	324	338	339	372	48	0.328767123
LM6	317	330	334	350	33	0.226027397
LM 10	284	291	305	340	56	0.383561644
LM11	227	242	242	260	33	0.226027397
LM13	235	244	247	280	45	0.308219178
LM16	265	286	294	340	75	0.51369863
LM20	161	161	170	190	29	0.198630137
LM25	210	220	221	248	38	0.260273973
LM28	181	191	194	230	49	0.335616438
Total	2204	2303	2346	2610	406	2.780821918
Mean	233	243	256	290	57	0.390410959

Table 2.2: Live body mass of animals from the low dose (LD) group

LD group	31/01/00	6/3/00	17/04/00	25/06/00	Total gain	Average daily gain
LM1	255	281	287	315	60	0.410958904
LM2	303	316	317	305	2	0.01369863
LM7	268	271	299	305	37	0.253424658
LM12	254	279	282	300	46	0.315068493
LM14	241	248	269	290	49	0.335616438
LM17	249	258	265	282	33	0.226027397
LM21	191	202	209	220	29	0.198630137
LM22	215	229	243	256	41	0.280821918
LM23	187	191	207	200	13	0.089041096
LM24	190	202	211	240	50	0.342465753
LM27	161	169	179	195	34	0.232876712
Total	2514	2646	2768	2908	394	2.698630137
Mean	229	241	252	264	35	0.239726027

Table 2.3: Live body mass of animals from the control group

Controls	31/01/00	6/3/00	17/04/00	25/06/00	Total gain	Average daily gain
LM4	401	419	414	440	39	0.267123288
LM5	266	286	303	315	49	0.335616438
LM8	298	320	319	350	52	0.356164384
LM9	249	271	274	300	51	0.349315068
LM18	215	256	268	290	75	0.51369863
LM26	164	183	191	220	56	0.383561644
Total	1593	1735	1769	1915	322	2.205479452
Mean	266	289	295	319	53	0.363013699

Table 2.4: Live body mass of the non-diseased animals

Number	31/01/00	6/3/00	17/04/00	25/06/00	Total gain	Average daily gain
LM4	401	419	414	440	39	0.267123288
LM5	266	286	303	315	49	0.335616438
LM7	268	271	299	305	37	0.253424658
LM8	298	320	319	350	52	0.356164384
LM9	249	271	274	300	51	0.349315068
LM12	254	279	282	300	46	0.315068493
LM18	215	256	268	290	75	0.51369863
LM23	187	191	207	200	13	0.089041096
LM26	164	183	191	220	56	0.383561644
LM27	161	169	179	195	34	0.232876712
Total	2463	39325	2736	2915	452	3.095890411
Mean	241	259	268	287	46	0.315068493

Table 2.5: Live body mass of the diseased animals

Number	31/01/00	6/3/00	17/04/00	25/06/00	Total gain	Average daily gain
LM1	255	281	287	315	60	0.410958904
LM2	303	316	317	305	2	0.01369863
LM3	324	338	339	372	48	0.328767123
LM6	317	330	334	350	33	0.226027397
LM 10	284	291	305	340	56	0.383561644
LM11	227	242	242	260	33	0.226027397
LM13	235	244	247	280	45	0.308219178
LM14	241	248	269	290	49	0.335616438
LM16	265	286	294	340	75	0.51369863
LM17	249	258	265	282	33	0.226027397
LM20	161	161	170	190	29	0.198630137
LM21	191	202	209	220	29	0.198630137
LM22	215	229	243	256	41	0.280821918
LM25	210	220	221	248	38	0.260273973
LM28	181	191	194	230	49	0.335616438
Total	3658	3837	3936	4278	620	4.246575342
Mean	236	247	259	285	49	0.335616438

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Table 3: The results of the intradermal tuberculin tests that were done 11 weeks after intratonsilar infection on all the animals of Infection model.

Number	Sex	Bovine 0hr	Bovine 72h	Avian 0hr	Avian 72hr	Results
Control group						
LM4	M	21.7	23.0	21.7	23.4	Neg.
LM5	M	17.2	19.5	16.0	19.4	Neg.
LM8	F	14.6	14.5	14.0	15.0	Neg.
LM9	F	13.4	15.4	11.6	15.5	Neg.
LM18	F	13.5	15.1	13.8	18.3	Neg.
LM26	M	10.1	9.6	10.7	12.8	Neg.
Low dose group						
LM1	M	13.3	31.4	13.0	15.5	Pos.
LM2	M	15.2	15.4	15.2	15.3	Neg.
LM7	F	15.5	16.2	15.9	16.6	Neg.
LM12	F	15.0	14.9	15.1	16.5	Neg.
LM14	F	12.6	16.6	12.2	13.4	Pos.
LM17	F	11.0	>33	10.8	13.1	Pos.
LM21	F	12.8	13.5	15.0	18.1	Neg.
LM22	M	12.1	>33	13.3	18.5	Pos.
LM23	M	10.6	12.5	14.2	17.0	Neg.
LM24	V	12.2	17.5	11.7	12.8	Pos.
LM27	M	11.4	16.7	10.7	12.7	Pos.
Number	**Sex**	**Bovine 0hr**	**Bovine 72hr**	**Avian 0hr**	**Avian 72hr**	**Result**
High dose group						
LM3	F	17.2	>33	16.4	20.5	Pos.
LM6	M	16.1	>33	16.7	17.4	Pos.
LM10	F	13.3	>33	12.8	17.3	Pos.
LM11	F	10.0	>33	11.0	12.5	Pos.
LM13	F	18.1	>33	17.3	18.4	Pos.
LM15	M	9.0	--	11.0	--	--
LM16	M	16.1	>33	14.8	22.2	Pos.
LM19	F	--	--	--	--	--
LM20	M	10.4	30.4	11.8	14.5	Pos.
LM25	M	13.0	19.7	12.6	14.4	Pos.
LM28	M	17.7	>33	11.5	14.8	Pos.

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Table 4: Results of the gamma-interferon assay over a six-month period starting on the day of intratonsilar infection.

Number	31/01/2000	06/03/2000	17/04/2000	08/05/2000	Last day	Results
Control group						
LM4	-	Eq	-	-	MR	Neg.
LM5	-	-	Invalid	-	-	Neg.
LM8	-	-	-	MR	-	Neg.
LM9	-	-	-	Av	Av	Neg.
LM18	-	-	-	-	-	Neg.
LM26	-	-	-	-	-	Neg.
Low dose group						
LM1	-	-	-	+	+	Pos.
LM2	-	-	-	-	-	Neg.
LM7	-	-	-	Invalid	-	Neg.
LM12	-	-	+	-	-	Neg.
LM14	-	-	-	-	-	Neg.
LM17	-	-	+	+	+	Pos.
LM21	-	-	Invalid	-	+	Pos.
LM22	-	-	MR	+	+	Pos.
LM23	-	-	-	-	-	Neg.
LM24	-	-	-	-	-	Neg.
LM27	-	-	-	MR	-	Neg.
High dose group						
LM3	-	-	+	+	+	Pos.
LM6	-	+	MR	+	+	Pos.
LM10	-	-	MR	+	+	Pos.
LM11	-	+	MR	+	+	Pos.
LM13	-	+	+	+	MR	Pos.
LM15	-	+	+			Pos.
LM16	-	-	-	+	+	Pos.
LM19	-	Suspect				Pos.
LM20	-	-	MR	+	+	Pos.
LM25	-	+	-	+	+	Pos.
LM28	-	+	Suspect	+	+	Pos.

Table 5: **ELISA results of the Infection model over a period of six months.**

Number	31/01/2000	06/03/2000	17/04/2000	08/05/2000	Last day	Results
Control group						
LM4	-	-	-	-	-	Neg.
LM5	-	-	-	-	-	Neg.
LM8	-	-	-	-	-	Neg.
LM9	-	-	-	-	-	Neg.
LM18	-	-	-	-	-	Neg.
LM26	-	-	-	-	-	Neg.
Low dose group						
LM1	-	-	-	+	+	Pos.
LM2	-	-	-	-	-	Neg.
LM7	-	-	-	-	-	Neg.
LM12	-	-	-	-	-	Neg.
LM14	-	-	-	-	-	Neg.
LM17	-	-	-	-	-	Neg.
LM21	-	-	-	-	-	Neg.
LM22	-	-	-	-	-	Neg.
LM23	-	-	-	-	-	Neg.
LM24	-	-	-	-	-	Neg.
LM27	-	-	-	-	-	Neg.
High dose group						
LM3	-	-	-	+	+	Pos.
LM6	-	-	-	+	+	Pos.
LM10	-	-	-	+	+	Pos.
LM11	-	-	-	+	+	Pos.
LM13	-	-	-	+	+	Pos.
LM15	-	-	-			Neg.
LM16	-	-	-	+	+	Pos.
LM19	-	-				Neg.
LM20	-	-	-	+	+	Pos.
LM25	-	-	-	-	-	Neg.
LM28	-	-	-	+	+	Pos.

6. The haematology values of the individual animals from the Infection model study

Table 6.1: Blood profiles of the Control group animals

LM 4	**31/1/00**	**6/3/00**	**17/4/00**	**8/5/00**	**5/7/00**	**Total**	**Mean**	**Stdev**
WBC	6	7.2	6.5	8	8.4	36.1	7.22	1.001
RBC	6.99	7.64	7.71	6.69	12.69	41.72	8.34	2.47
Hgb g/dl	9.8	10.4	11	9.4	18.2	58.8	11.8	3.65
Hct %	27.4	29.9	29.8	25.3	49.8	162.2	32.4	9.89
MCV fl	39.2	39.1	38.6	37.8	39.2	193.9	38.8	0.60
MCH pg	14.1	13.7	14.3	14	14.3	70.4	14.1	0.25
MCHC g/d	35.9	34.9	36.9	37	36.6	181.3	36.3	0.87
PLT 10^3	589	756	941	847	1107	4240	848	194
Lymph %	88	89.9	71.7	90.7	75.7	416	83.2	8.84
Lym 10^3	5.3	6.5	4.7	7.2	6.3	30	6	0.99

LM 5	31/1/00	6/3/00	17/4/00	8/5/00	1/6/00	Total	Mean	Stdev
WBC	9.4	9.7	7.6	7.6	9.6	43.9	8.8	1.08
RBC	9.18	9.92	10.81	10.99	12.05	52.95	10.59	1.09
Hgb g/dl	12	13	14.5	14.3	16.8	70.6	14.1	1.81
Hct %	34.7	36.7	40.1	40.6	45.5	197.6	39.5	4.14
MCV fl	37.8	37	37.1	36.9	37.7	186.5	37.3	0.42
MCH pg	13	13.1	13.4	13	14	66.5	13.3	0.42
MCHC g/d	34.5	35.5	36.2	35.2	37.1	178.5	35.7	0.99
PLT 10^3	941	1232	995	1102	1191	5461	1092	124
Lymph %	87.4	87.2	80.9	88.9	70.2	414.6	82.9	7.75
Lym 10^3	8.2	8.4	6.2	6.7	6.7	36.2	7.2	0.99

LM 8	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	8.8	8.2	8.2	8.6	9.3	43.1	8.6	0.46
RBC	9.42	9.2	9.04	9.91	12.01	49.58	9.92	1.22
Hgb g/dl	14.6	14.2	14.5	15.6	19.6	78.5	15.7	2.24
Hct %	42.2	41.1	40.4	44.4	57.1	225.2	45	6.91
MCV fl	44.8	44.7	44.7	44.7	47.5	226.4	45.3	1.24
MCH pg	15.5	15.4	16	15.7	16.3	78.9	15.8	0.37
MCHC g/d	34.6	34.5	35.8	35.1	34.3	174.3	34.9	0.60
PLT 10^3	462	415	390	355	409	2031	406	39
Lymph %	86.8	82.2	70.6	90.1	75.7	405.4	81.1	7.97
Lym 10^3	7.6	6.8	5.8	7.8	7	35	7	0.79

LM 9	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	11.3	10.7	8.3	8.4	8.6	47.3	9.5	1.43
RBC	11.15	11.87	10.85	12.02	13.54	59.43	11.89	1.05
Hgb g/dl	14.8	15	14.4	15.9	17.6	77.7	15.5	1.28
Hct %	41.6	42.8	39	44.3	51.6	219.3	43.9	4.74
MCV fl	37.3	36.1	35.9	36.8	38.1	184.2	36.8	0.9
MCH pg	13.3	12.6	13.3	13.2	13	65.4	13.1	0.29
MCHC g/d	35.6	35	37	36	34	177.6	35.5	1.12
PLT 10^3	1428	1724	1537	1455	1410	7554	1511	129
Lymph %	88.1	86.6	73.9	90	90	428.6	85.7	6.76
Lym 10^3	9.9	9.3	6.1	7.6	7.8	40.7	8.1	1.50

LM 18	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	8	9.6	9.6	7.7	8.9	43.8	8.8	0.88
RBC	8.58	9.57	9.88	11.63	14.34	54	10.8	2.26
Hgb g/dl	11	12.1	13	15.2	19.2	70.5	14.1	3.24
Hct %	32.3	35	35.5	42.4	56.5	201.7	40.3	9.77
MCV fl	37.6	36.6	35.9	36.5	39.4	186	37.2	1.37
MCH pg	12.8	12.7	13.2	13.1	13.4	65.2	13	0.29
MCHC g/d	34.1	34.7	36.6	35.8	33.9	175.1	35	1.15
PLT 10^3	906	1274	1431	1643	1446	6700	1340	276
Lymph %	86.7	90.8	84.2	92.7	89.6	444	88.8	3.37
Lym 10^3	6.9	8.7	8.1	10.8	8	42.5	8.5	1.44

LM 26	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	4.5	6	4.4	6.1	8.5	29.5	5.9	1.66
RBC	9.18	10.39	10.75	12.09	14.52	56.93	11.34	2.04
Hgb g/dl	9.5	11	11.7	13.2	15.8	61.2	12.2	2.39
Hct %	27.6	31	31.8	35.3	44.9	170.6	34.1	6.62
MCV fl	30.1	29.9	29.6	29.2	30.9	149.7	30	0.63
MCH pg	10.3	10.6	10.8	10.9	10.9	53.5	10.7	0.25
MCHC g/d	34.4	35.4	36.7	37.3	35.2	179	35.8	1.18
PLT 10^3	3951	5100	5048	5756	5679	25534	5107	723
Lymph %	93.4	87.4	78.5	93.5	85.1	437.9	87.6	6.28
Lym 10^3	4.2	5.2	3.4	5.7	7.2	25.7	5.1	1.46

Table 6.2: Blood profiles of the Low dose (LD) group animals

LM 1	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	12.1	13.5	11.2	12.8	11.1	60.7	12.1	1.03
RBC	10.27	12.22	7.86	11.99	15.93	58.27	11.65	2.96
Hgb g/dl	12.2	14.4	15.1	14.4	19.3	75.4	15.1	2.6
Hct %	36	42.4	32.2	41.2	56.5	208.3	41.7	9.25
MCV fl	35.1	34.7	41	34.4	35.5	180.7	36.1	2.75
MCH pg	11.9	11.8	19.2	12	12.1	67	13.4	3.24
MCHC g/d	33.9	34	46.9	34.9	34.1	183.8	36.8	5.68
PLT 10^3	1672	2399	2882	2063	2706	11722	2344	488
Lymph %	86.5	85.4	75	90.5	87.2	424.6	84.9	5.86
Lym 10^3	10.5	11.6	8.4	11.6	9.7	51.8	10.4	1.36

LM 2	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	7.9	7.7	6.4	7.2	9	38.2	7.6	0.96
RBC	10.25	11.58	10.98	10.86	12.82	56.49	11.3	0.97
Hgb g/dl	13.3	14.9	14.2	14.2	16.5	73.1	14.6	1.19
Hct %	38.1	42.4	38.9	39.1	46.9	205.4	41.1	3.65
MCV fl	37.2	36.7	35.5	36	36.6	182	36.4	0.66
MCH pg	13	12.9	12.9	13.1	12.9	64.8	13	0.09
MCHC g/d	34.9	35.1	36.5	36.3	35.2	178	35.6	0.74
PLT 10^3	1163	1501	1757	1510	2047	7978	1596	329
Lymph %	84.8	85.8	80	89.8	86.1	426.5	85.3	3.52
Lym 10^3	6.7	6.6	5.1	6.5	7.7	32.6	6.5	0.93

LM 7	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	9.9	9.2	8.3	8.1	12.6	48.1	9.6	1.82
RBC	8.88	10.28	9.9	9.99	13.52	52.57	10.51	1.76
Hgb g/dl	11.4	13.8	13.9	13.7	18.1	70.9	14.2	2.43
Hct %	32.2	38.6	37.9	37.3	52	198	39.6	7.38
MCV fl	36.2	37.6	38.3	37.3	38.5	187.9	37.6	0.91
MCH pg	12.8	13.4	14	13.7	13.4	67.3	13.5	0.44
MCHC g/d	35.3	35.7	36.7	36.7	34.8	179.2	35.8	0.85
PLT 10^3	1315	1528	1170	1140	1414	6567	1313	163
Lymph %	89.1	91.1	85.6	93.2	91.6	450.6	90.1	2.92
Lym 10^3	8.8	8.4	7.1	7.6	11.5	43.4	8.7	1.71

LM 12	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	9	9.1	7.8	7.9	12.3	46.1	9.2	1.82
RBC	10.72	12.95	11.68	12.2	13.42	60.97	12.19	1.06
Hgb g/dl	13.8	16.1	15.3	15.4	18.2	78.8	15.8	1.6
Hct %	39.1	45.9	41.4	43.9	52.2	222.5	44.5	5.01
MCV fl	36.4	35.5	35.5	36.5	38.9	182.8	36.6	1.39
MCH pg	12.9	12.4	13.1	12.8	13.5	64.7	12.9	0.40
MCHC g/d	35.3	35	36.9	35.2	34.8	177.2	35.4	0.84
PLT 10^3	1475	2274	2058	1703	1386	8896	1779	379
Lymph %	90.9	89.5	73.9	88	84.4	426.7	85.3	6.84
Lym 10^3	8.2	8.1	5.7	6.9	10.4	39.3	7.9	1.75

LM 14	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	9	9	8.6	10	8.9	45.5	9.1	0.53
RBC	9.96	11.38	10.78	11.92	12.77	56.81	11.36	1.07
Hgb g/dl	12.1	14.8	14.3	15.4	16.8	73.4	14.7	1.72
Hct %	35.2	41.6	38.9	43.5	48.5	207.7	41.5	4.99
MCV fl	35.3	36.6	36.1	36.5	38	182.5	36.5	0.98
MCH pg	12.1	13	13.2	12.9	13.1	64.3	12.9	0.44
MCHC g/d	34.4	35.5	36.6	35.5	34.5	176.5	35.3	0.9
PLT 10^3	1853	2018	1844	1866	1599	9180	1836	150
Lymph %	82.2	87.2	78.4	91.7	80.7	420.2	84	5.36
Lym 10^3	7.4	7.8	6.7	9.2	7.1	38.2	7.6	0.96

LM 17	31/1/00	6/3/00	17/4/00	8/5/00	1/6/00	Total	Mean	Stdev
WBC	5.9	6.7	6.8	7.8	10.9	38.1	7.6	1.95
RBC	8.05	8.41	8.76	9.25	11.72	46.19	9.24	1.46
Hgb g/dl	11.7	11.7	12.6	12.7	17.9	66.6	13.3	2.6
Hct %	32.8	33.1	33.9	35.9	48.2	183.9	36.8	6.5
MCV fl	40.8	39.4	39.7	38.8	41.2	199.9	40	1
MCH pg	14.6	13.9	14.4	13.7	15.3	71.9	14.4	0.63
MCHC g/d	35.8	35.2	37.3	35.4	37	180.7	36.1	0.95
PLT 10^3	589	746	682	780	769	3566	713	79
Lymph %	88.7	93.6	83.8	92.9	87.4	446.4	89.3	4.05
Lym 10^3	5.2	6.3	5.7	7.3	9.5	34	6.8	1.7

LM 21	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC		4.1	4.5	3.8	4.9	17.3	4.3	0.48
RBC	7.32	6.41	6.71	6.89	11.39	38.72	7.74	2.06
Hgb g/dl	8.9	8.4	8.9	9.2	15	50.4	10.1	2.77
Hct %	27.8	24.4	25	26	46	149.2	29.8	9.13
MCV fl	38	38.1	37.3	37.8	40.3	191.5	38.3	1.16
MCH pg	12.1	13.1	13.3	13.3	13.1	64.9	13	0.5
MCHC g/d	32	34.3	35.8	35.3	32.5	169.9	34	1.68
PLT 10^3	609	716	604	692	825	3446	689	91
Lymph %		94.9	88.7	93	94.7	371.3	92.8	2.88
Lym 10^3		3.8	4	3.5	4.6	15.9	4	0.46

LM 22	31/1/00	6/3/00	17/4/00	8/5/00	1/6/00	Total	Mean	Stdev
WBC	10.4	8.3	9.2	8.4	9	45.3	9.1	0.84
RBC	10.97	11.68	11.32	11.77	13.45	59.19	11.84	0.96
Hgb g/dl	11.7	12.4	12.8	12.9	16.1	65.9	13.2	1.7
Hct %	35.5	36.9	36	37.6	45	191	38.2	3.89
MCV fl	32.3	31.6	31.8	32	33.5	161.2	32.2	0.75
MCH pg	10.7	10.6	11.3	10.9	12	55.5	11.1	0.57
MCHC g/d	33.1	33.6	35.5	34.2	35.8	172.2	34.4	1.18
PLT 10^3	3275	4187	3510	3494	3095	17561	3512	414
Lymph %	83.4	92.2	75.1	90.6	59	400.3	80.1	13.58
Lym 10^3	8.7	7.6	6.9	7.6	5.3	36.1	7.2	1.25

LM 23	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC		4	4.2	4.2	7.7	20.1	5	1.79
RBC	10.2	11.61	11.15	12.23	11.63	56.82	11.36	0.76
Hgb g/dl	11.3	14.2	14.1	12.5	14.2	66.3	13.3	1.31
Hct %	34.9	40.9	39.3	43.9	42.8	201.8	40.4	3.53
MCV fl	34.8	35.2	35.2	35.8	36.8	177.8	35.6	0.78
MCH pg	11.2	12.2	12.6	12.6	12.2	60.8	12.2	0.57
MCHC g/d	32.3	34.6	35.9	35.3	33.1	171.2	34.2	1.51
PLT 10^3	1967	2469	2291	2100	2029	10856	2171	206
Lymph %		90	86.1	91.6	82.4	350.1	87.5	4.12
Lym 10^3		3.6	3.6	3.8	6.3	17.3	4.3	1.32

LM 24	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC		5	5.8	6.2	10	27	6.8	2.22
RBC	9.71	11.07	11.52	12.09	13.02	57.41	11.48	1.23
Hgb g/dl	10.1	12.3	13.9	13.4	14.7	64.4	12.9	1.78
Hct %	30.8	34.3	35.6	37.4	41.8	179.9	36	4.05
MCV fl	31.7	31	30.9	30.9	32.1	156.6	31.3	0.55
MCH pg	10.4	11.1	12.1	11	11.3	55.9	11.2	0.61
MCHC g/d	32.7	35.7	39.1	35.8	35.2	178.5	35.7	2.28
PLT 10^3	3025	4421	4526	4507	4477	20956	4191	653
Lymph %		89.1	76.6	89.1	77.3	332.1	83	7.02
Lym 10^3		4.5	4.4	5.5	7.7	22.1	5.5	1.53

LM 27	31/1/00	6/3/00	17/4/00	8/5/00	5/7/00	Total	Mean	Stdev
WBC	5.2	5.3	4.6	5.2	4.1	24.4	4.9	0.52
RBC	9.83	10.29	9.75	10.21	12.38	52.46	10.5	1.08
Hgb g/dl	10.4	11.4	11.5	12	13.9	59.2	11.8	1.29
Hct %	29.5	32.5	31.6	33.3	41.3	168.2	33.6	4.51
MCV fl	30	31.6	32.3	32.6	33.3	159.8	32	1.25
MCH pg	10.6	11.1	11.8	11.8	11.3	56.6	11.3	0.51
MCHC g/d	35.4	35.2	36.4	36.1	33.8	176.9	35.4	1.01
PLT 10^3	453	4298	3456	3100	3084	14391	2878	1442
Lymph %	85.9	93.1	88.2	94.2	91.6	453	90.6	3.47
Lym 10^3	4.5	5	4.1	4.9	3.7	22.2	4.4	0.55

Table 6.3: Blood profiles of the High dose (HD) group animals

LM 3	31/1/00	6/3/00	17/4/00	8/5/00	1/6/00	Total	Mean	Stdev
WBC	9.8	9.5	7.5	7.8	9.8	44.4	8.9	1.13
RBC	9.84	10.23	9.16	10.88	13.18	53.29	10.66	1.54
Hgb g/dl	12.5	13	12.1	13.9	18.3	69.8	14	2.52
Hct %	36.4	37.2	32.4	39.5	49.7	195.2	39	6.49
MCV fl	37	36.4	35.4	36.3	37.7	182.8	36.6	0.86
MCH pg	12.7	12.7	13.2	12.8	13.9	65.3	13.1	0.51
MCHC g/d	34.3	34.9	37.1	35.2	36.9	178.4	35.7	1.25
PLT 10^3	1202	1577	1566	1507	1467	7319	1464	153
Lymph %	81.9	65.1	71.4	92.4	65.1	375.9	75.2	11.82
Lym 10^3	8	8.1	5.4	7.2	6.4	35.1	7	1.14

LM 6	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	8.5	6.5	6.7	5.8	8.6	36.1	7.2	1.26
RBC	7.81	9.95	9.05	10.25	13.58	50.64	10.13	2.15
Hgb g/dl	8.7	11.7	10.9	11.8	16.3	59.4	11.9	2.77
Hct %	26.6	33.4	29.7	33.8	48.4	171.9	34.4	8.37
MCV fl	34.1	33.6	32.8	33	35.7	169.2	33.8	1.16
MCH pg	11.1	11.7	12.1	11.6	12	58.5	11.7	0.39
MCHC g/d	32.7	35	36.7	35	33.6	173	34.6	1.53
PLT 10^3	1460	2378	2068	2216	2463	10585	2117	397
Lymph %	86.7	87.9	79.6	93.1	86.1	433.4	86.7	4.83
Lym 10^3	7.3	5.7	5.4	5.4	7.4	31.2	6.2	1.02

LM 10	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	13.7	6	7.2	6.9	7.7	41.5	8.3	3.08
RBC	9.18	8.74	8.49	8.86	12.49	47.76	9.55	1.66
Hgb g/dl	12.3	11.4	11	11.1	15.8	61.6	12.3	2.01
Hct %	35	32.6	30	31.3	47.6	176.5	35.3	7.12
MCV fl	36.1	37.3	35.4	35.3	38.1	182.2	36.4	1.22
MCH pg	13.4	13.1	13	12.5	12.6	64.6	12.9	0.37
MCHC g/d	35.1	35.1	36.7	35.4	33.1	175.4	35.1	1.29
PLT 10^3	1276	1195	1379	1506	1776	7132	1426	227
Lymph %	87.3	87.7	80.2	92.9	78.2	426.3	85.3	6
Lym 10^3	12	5.3	5.8	6.4	6	35.5	7.1	2.77

LM 11	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	8.5	5.8	6.7	5.2	7	33.2	6.6	1.26
RBC	8.37	9.84	9.37	9.11	13.32	50.01	10	1.93
Hgb g/dl	11.2	13.1	13.1	12.2	17.8	67.4	13.5	2.54
Hct %	31.4	37.5	35.5	34.3	53.9	192.6	38.5	8.88
MCV fl	37.4	38.1	37.9	37.7	40.4	191.5	38.3	1.2
MCH pg	13.3	13.3	14	13.4	13.4	67.4	13.5	0.29
MCHC g/d	35.6	34.9	36.9	35.6	33.1	176.1	35.2	1.39
PLT 10^3	1207	1393	1006	984	923	5513	1103	194
Lymph %	89.2	91.8	72.1	92.7	81.1	426.9	85.4	8.72
Lym 10^3	7.6	5.3	4.8	4.8	5.7	28.2	5.6	1.16

LM 13	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	9.3	9.6	8.5	7.7	13.1	48.2	9.6	2.07
RBC	9.05	11.53	11.07	10.29	12.66	54.6	10.92	1.35
Hgb g/dl	11.4	14.6	15.2	13.2	16.9	71.3	14.3	2.08
Hct %	34.9	44.2	43	39.4	51.3	212.8	42.6	6.08
MCV fl	38.5	38.3	38.8	38.2	40.5	194.3	38.9	0.94
MCH pg	12.6	12.7	13.7	12.8	13.3	65.1	13	0.47
MCHC g/d	32.7	33.1	35.2	33.5	32.9	167.4	33.5	1.01
PLT 10^3	1095	1404	1189	1219	1317	6224	1245	119
Lymph %	87.7	89.4	76.4	94.8	82.4	430.7	86.1	7.02
Lym 10^3	8.2	8.6	6.5	7.3	10.8	41.4	8.3	1.63

LM 15	31/1/00	6/3/00	17/4/00			Total	Mean	Stdev
WBC	9.2	6.8	6.8			22.8	7.6	1.39
RBC	10.05	10.11	9.55			29.71	9.9	0.31
Hgb g/dl	12.7	12.3	11.5			36.5	12.2	0.61
Hct %	36.7	36.1	31.9			104.7	34.9	2.62
MCV fl	36.5	35.7	33.4			105.6	35.2	1.61
MCH pg	12.6	12.1	12.1			36.8	12.3	0.29
MCHC g/d	34.5	34	36.1			104.6	34.9	1.1
PLT 10^3	1311	1497	1891			4699	1566	296
Lymph %	85.1	89.7	73.9			248.7	49.7	8.13
Lym 10^3	7.6	6.1	5			18.7	6.2	1.31

LM 16	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC	7.3	6.5	6.9	7.3	8.7	36.7	7.3	0.83
RBC	9.33	10.07	10.46	12.09	14.07	56.02	11.2	1.89
Hgb g/dl	11	12	13	14.7	16.9	67.6	13.5	2.33
Hct %	32.1	34.3	35.7	41.3	51.5	194.9	39	7.78
MCV fl	34.4	34.1	34.1	34.1	36.7	173.4	34.7	1.14
MCH pg	11.8	11.9	12.5	12.1	12	60.3	12.1	0.27
MCHC g/d	34.5	34.9	36.4	35.5	32.7	174	34.8	1.37
PLT 10^3	1690	2113	1948	2273	2042	10066	2013	216
Lymph %	87.8	93	84.1	91	87.7	443.6	88.7	3.42
Lym 10^3	6.4	6.1	5.8	6.6	7.6	32.5	6.5	0.69

LM 19	31/1/00	6/3/00				Total	Mean	Stdev
WBC	5.9	4.9				10.8	5.4	0.71
RBC	6.78	6.8				13.58	6.8	0.01
Hgb g/dl	8.9	9.1				18	9	0.14
Hct %	26.6	26.5				53.1	26.6	0.07
MCV fl	39.2	38.9				78.1	39.1	0.21
MCH pg	13.2	13.3				26.5	13.3	0.07
MCHC g/d	33.6	34.3				67.9	34	0.49
PLT 10^3	826	808				1634	817	13
Lymph %	85	93.8				178.8	89.4	6.22
Lym 10^3	5	4.5				9.5	4.8	0.35

LM 20	31/1/00	6/3/00	17/4/00	8/5/00	28/6/00	Total	Mean	Stdev
WBC		6.3	5.5	6.4	6.9	25.1	6.3	0.58
RBC	7.82	8.94	10.85	11.67	14.28	53.56	10.71	2.51
Hgb g/dl	8.5	10.8	12.9	13.3	15.7	61.2	12.2	2.72
Hct %	25.3	31.4	35.8	37.6	46.7	176.8	35.4	7.92
MCV fl	32.3	35.1	33	32.2	32.7	165.3	33.1	1.18
MCH pg	10.9	12.1	11.9	11.4	11	57.3	11.5	0.53
MCHC g/d	33.7	34.4	36.1	35.4	33.6	173.2	34.6	1.09
PLT 10^3	2823	2283	2623	3395	4255	15379	3076	773
Lymph %		93.6	83.3	93.3	89	359.2	89.8	4.82
Lym 10^3		5.9	4.5	6	6.2	22.6	5.7	0.78

LM 25	31/1/00	6/3/00	17/4/00	8/5/00	1/6/00	Total	Mean	Stdev
WBC	6	7.5	7.2	6.7	7.4	34.8	7	0.62
RBC	10.61	12.79	11.08	12.81	13.67	60.96	12.13	1.29
Hgb g/dl	11.6	14.7	12.9	14.6	17	70.8	14.2	2.04
Hct %	35.9	43.1	36.4	42.7	47.5	205.6	41.1	4.92
MCV fl	33.8	33.7	32.8	33.4	34.7	168.4	33.7	0.69
MCH pg	11	11.5	11.6	11.4	12.4	57.9	11.6	0.59
MCHC g/d	32.4	34.1	35.4	34.1	35.7	171.7	34.3	1.31
PLT 10^3	2417	3069	2750	2952	2527	13715	2743	275
Lymph %	92.2	91.6	82.6	92.4	78.3	437.1	87.4	6.55
Lym 10^3	5.6	6.9	6	6.2	5.8	30.5	6.1	0.5

LM 28	31/1/00	6/3/00	17/4/00	8/5/00	1/6/00	Total	Mean	Stdev
WBC	6.7	8.6	7.1	6.3	6.2	34.9	7	0.97
RBC	8.1	9.06	9.14	8.97	11.68	46.95	9.39	1.35
Hgb g/dl	10.8	12.4	13.6	12.4	16	65.2	13	1.93
Hct %	31.7	35.7	36.2	35.2	47.6	186.4	37.3	6.03
MCV fl	39.2	39.4	39.6	39.3	40.8	198.3	39.7	0.65
MCH pg	13.4	13.7	14.9	13.9	13.7	69.6	13.9	0.58
MCHC g/d	34.1	34.9	37.7	35.2	33.5	175.4	35.1	1.61
PLT 10^3	998	803	578	860	1083	4322	864	195
Lymph %	93.6	91.2	85	96.4	76.2	442.4	88.5	8.05
Lym 10^3	6.2	7.9	6	6.1	4.7	30.9	6.2	1.14

Table 6.4: Mean haematological values of healthy animals

Nr.	WBC	RBC	Hgb g/dl	Hct %	MCV fl	MCH pg	MCHC g/d	Platelet 10^3	Lymph %	Lym 10^3
LM4	7.20	8.34	11.80	32.40	38.80	14.10	36.30	848	83.20	6.00
LM5	8.80	10.59	14.10	39.50	37.30	13.30	35.70	1092	92.90	7.20
LM7	9.60	10.51	14.20	39.60	37.60	13.50	35.80	1313	90.10	8.70
LM8	8.60	9.92	15.70	45.00	45.30	15.80	34.90	406	81.10	7.00
LM9	9.50	11.89	15.50	43.90	36.80	13.10	35.50	1511	85.70	8.10
LM12	9.20	12.19	15.80	44.50	36.60	12.90	35.40	1779	85.30	7.90
LM18	8.80	10.80	14.10	40.30	37.20	13.00	35.00	1340	88.80	8.50
LM23	5.00	11.36	13.30	40.40	35.60	12.20	34.20	2171	87.50	4.30
LM24	6.80	11.48	12.90	36.00	31.30	11.20	35.70	4191	83.00	5.50
LM26	5.90	11.34	12.20	34.10	30.00	10.70	35.80	5107	87.60	5.10
LM27	4.90	10.50	11.80	33.60	32.00	11.30	35.40	2878	90.60	4.40
Total	84.30	118.92	151.40	429.30	398.50	141.10	389.70	22636	955.80	72.70
Mean	7.66	10.81	13.76	39.03	36.23	12.83	35.43	2058	86.89	6.61
SDEV	1.78	1.06	1.5	4.47	4.18	1.45	0.56	1453	3.62	1.63

Table 6.5: Mean haematological values of diseased animals

Nr.	WBC	RBC	Hgb g/dl	Hct %	MCV fl	MCH pg	MCHC g/d	Platelet 10^3	Lymph %	Lymph 10^3
LM1	12.10	11.65	15.10	41.70	36.10	13.40	36.80	2344	84.90	10.40
LM2	7.60	11.30	14.60	41.10	36.40	13.00	35.60	1596	85.30	6.50
LM3	8.90	10.66	14.00	39.00	36.60	13.10	35.70	1464	75.20	7.00
LM6	7.20	10.13	11.90	34.40	33.80	11.70	34.60	2117	86.70	6.20
LM10	8.30	9.55	12.30	35.30	36.40	12.90	35.10	1426	85.30	7.10
LM11	6.60	10.00	13.50	38.50	38.30	13.50	35.20	1103	85.40	5.60
LM13	9.60	10.92	14.30	42.60	38.90	13.00	33.50	1245	86.10	8.30
LM14	9.10	11.36	14.70	41.50	36.50	12.90	35.30	1836	84.00	7.60
LM15	7.60	9.90	12.20	34.90	35.20	12.30	34.90	1566	82.90	6.20
LM16	7.30	11.20	13.50	39.00	34.70	12.10	34.80	2013	88.70	6.50
LM17	7.60	9.24	13.30	36.80	40.00	14.40	36.10	713	89.30	6.80
LM19	5.40	6.80	9.00	26.60	39.10	13.30	34.00	817	89.40	4.80
LM20	6.30	10.71	12.20	35.40	33.10	11.50	34.60	3076	89.80	5.70
LM21	4.30	7.74	10.10	29.80	38.30	13.00	34.00	689	92.80	4.00
LM22	9.10	11.84	13.20	38.20	32.20	11.10	34.40	3512	80.10	7.20
LM25	7.00	12.13	14.20	41.10	33.70	11.60	34.30	2743	87.40	6.10
LM28	7.00	9.39	13.00	37.30	39.70	13.90	35.10	864	88.50	6.20
Total	131.00	174.52	221.10	633.20	619.00	216.70	594.00	29124	1461.80	112.20
Mean	7.71	10.26	13.01	37.25	36.41	12.75	34.94	1713	85.99	6.6
SDEV	1.77	1.43	1.62	4.29	2.39	0.90	0.82	835	4.08	1.41

Table 7: Macroscopic tuberculous lesions observed when the buffalo calves from the Infection model were presented for necropsy.

Number	Tonsil	L. retro	Head	Thorax	Lung	Other	Grading
Control group							**0**
LM4	-	-	-	-	-	-	0
LM5	-	-	-	-	-	-	0
LM8	-	-	-	-	-	-	0
LM9	-	-	-	-	-	-	0
LM18	-	-	-	-	-	-	0
LM26	-	-	-	-	-		0
Low Dose group							**8**
LM1	-	+	-	+	-	-	3
LM2	-	-	-	-	-	-	0
LM7	-	-	-	-	-	-	0
LM12	-	-	-	-	-	-	0
LM14	-	-	-	-	-	-	0
LM17	-	+	-	-	-	-	2
LM21	-	+	-	-	-	-	1
LM22	-	+	-	-	-	-	2
LM23	-	-	-	-	-	-	0
LM24	-	-	-	-	-	-	0
LM27	-	-	-	-	-	-	0
Medium Dose group							**17**
LM3	-	+	-	-	-	-	2
LM6	-	+	-	-	-	-	2
LM10	-	+	-	+	-	-	3
LM11	-	+	-	-	-	-	2
LM13	-	+	-	-	-	-	2
LM15	-	+	-	-	-	-	2
LM16	-	-	-	-	-	-	0
LM19	-	-	-	-	-	-	0
LM20	-	+	-	-	-	-	1
LM25	-	+	-	-	-	-	1
LM28	-	+	-	-	-	-	2

ANNEXURE II

Table 1: Table of BCG Vaccine study animals

Control Group			Vaccinated Group		
Animal nr.	**Sex**	**Buffalo herd**	**Animal nr.**	**Sex**	**Buffalo herd**
LM 2/z	M	Malahlapanga	LM 1/z	M	Malahlapanga
LM 4/z	F	"	LM 3/z	F	"
LM 5/z	F	"	LM 10/z	M	Shipande
LM 7/z	F	"	LM 14/z	F	"
LM 8/z	F	Shipande	LM 16/z	F	"
LM 9/z	F	"	LM 18/z	M	Klopperfontein
LM 11/z	F	"	LM 19/z	F	"
LM 12/z	F	"	LM 21/z	M	"
LM 15/z	F	"	LM 23/z	F	"
LM 20/z	F	Tsombeyeni	LM 25/z	F	Tsombeyeni
LM 24/z	M	"	LM 27/z	F	"
LM 26/z	M	"	LM 29/z	F	"
LM 28/z	F	"	LM 30/z	M	"
LM 31/z	M	"	LM 32/z	F	Gadzingwe
LM 33/z	M	Gadzingwe	LM 35/z	F	"

Table 2.1: Mean body mass values of the control and vaccine group animals.

	10/10/00	04/12/00	15/01/01	03/04/01	08/05/01	07/06/01	13/07/01	07/08/01	30/10/01
Control	233kg	242kg	258kg	277kg	276kg	277kg	289kg	297kg	302kg
Vaccine	238kg	250kg	258kg	280kg	278kg	277kg	288kg	297kg	309kg

Table 2.2: Mean body mass values of diseased and non-diseased animals.

	10/10/00	04/12/00	15/01/01	03/04/01	08/05/01	07/06/01	13/07/01	07/08/01	30/10/01
Diseased	232	242	254	274	274	274	284	293	300
Non-diseased	251	267	279	301	293	289	312	317	327

Table 2.3: Mean gain in body mass (kg) and average daily gains (kg/day) of control, vaccinated, diseased and non-diseased animals.

	Control	Vaccinated	Diseased	Non-diseased
Mean gain (kg)	79	73	75	76
Average daily gain (kg/day)	0.202	0.191	0.196	0.198

Table 2.4: Weight table of the control group animals of the BCG vaccine study

	10/10/00	4/12/00	15/1/01	3/4/01	8/5/01	7/6/01	13/7/01	7/8/01	30/10/01	Total gain	Average gain
LM 2/z	333	350	376	393	389	350	392	403	425	92	0.23896104
LM 4/z	233	252	276	294	285	295	290	308	307	74	0.19220779
LM 5/z	173	180	191	216	220	221	234	235	260	87	0.22597403
LM 7/z	204	201	210	230	232	240	243	255	260	56	0.14545455
LM 8/z	191	204	224	236	249	272	266	267	278	87	0.22597403
LM 9/z	184	186	192	222	213	231	244	250	257	73	0.18961039
LM 11/z	244	256	280	311	289	305	346	328	338	94	0.24415584
LM 12/z	201	222	235	242	250	256	248	264			
LM 15/z	292	310	338	362	346	327	360	373	395	103	0.26753247
LM 20/z	267	285	305	315	311	326	326	340			
LM 24/z	219	216	229	248	251	252	255	270	295	76	0.1974026
LM 28/z	326	338	351	375	370	344	379	387			
LM 31/z	178	184	201	221	235	230	234	239	263	85	0.22077922
LM 33/z	216	207	205	223	229	230	233	241	245	29	0.07532468
Total	3261	3391	3613	3888	3869	3879	4050	4160	3323	856	2.22337662
Mean	**233**	**242**	**258**	**277**	**276**	**277**	**289**	**297**	**302**	**79**	**0.20212515**

Table 2.5: Weight table of the vaccine group of the BCG study

	10/10/00	4/12/00	15/1/01	3/4/01	8/5/01	7/6/01	13/7/01	7/8/01	30/10/01	Total gain	Average gain
LM 1/z	297	311	345	388	382	360	388	400	440	143	0.37142857
LM 3/z	283	309	321	333	336	312	341	353	360	77	0.2
LM 10/z	211	209	226	236	253	251	258	270			
LM 14/z	267	276	280	304	295	303	286	301			
LM 16/z	163	163	173	192	202	200	207	219	230	67	0.17402597
LM 18/z	174	180	184	205	207	208	216	225	235	61	0.15844156
LM 19/z	262	293	293	314	308	315	328	333			
LM 21/z	257	278	289	323	304	320	327	346	360	103	0.26753247
LM 23/z	262	281	272	294	288	295	298	304	308	46	0.11948052
LM 25/z	192	198	212	221	235	239	250	263	300	108	0.28051948
LM 27/z	299	306	311	336	321	305	342	346	341	42	0.10909091
LM 29/z	254	273	277	298	300	294	293	305	312	58	0.15064935
LM 30/z	225	241	243	284	284	287	296	306	345	120	0.31168831
LM 32/z	223	231	227	244	240	239	250	243	215	-8	-0.02077922
LM 35/z	196	209	221	232	217	223	235	246	260	64	0.16623377
Total	3565	3758	3874	4204	4172	4151	4315	4460	3706	881	2.28831169
Mean	**238**	**250**	**258**	**280**	**278**	**277**	**288**	**297**	**309**	**73**	**0.19069264**

Table 2.6: Diseased calves from the BCG study

	10/10/00	4/12/00	15/1/01	3/4/01	8/5/01	7/6/01	13/7/01	7/8/01	30/10/01	Total gain	Average gain
LM 1/z	297	311	345	388	382	360	388	400	440	143	0.37142857
LM 2/z	333	350	376	393	389	350	392	403	425	92	0.23896104
LM 4/z	233	252	276	294	285	295	290	308	307	74	0.19220779
LM 5/z	173	180	191	216	220	221	234	235	260	87	0.22597403
LM 7/z	204	201	210	230	232	240	243	255	260	56	0.14545455
LM 8/z	191	204	224	236	249	272	266	267	278	87	0.22597403
LM 9/z	184	186	192	222	213	231	244	250	257	73	0.18961039
LM 10/z	211	209	226	236	253	251	258	270			
LM 12/z	201	222	235	242	250	256	248	264			
LM 14/z	267	276	280	304	295	303	286	301			

LM 16/z	163	163	173	192	202	200	207	219	230	67	0.17402597
LM 19/z	262	293	293	314	308	315	328	333			
LM 20/z	267	285	305	315	311	326	326	340			
LM 21/z	257	278	289	323	304	320	327	346	360	103	0.26753247
LM 24/z	219	216	229	248	251	252	255	270	295	76	0.1974026
LM 25/z	192	198	212	221	235	239	250	263	300	108	0.28051948
LM 27/z	299	306	311	336	321	305	342	346	341	42	0.10909091
LM 28/z	326	338	351	375	370	344	379	387			
LM 29/z	254	273	277	298	300	294	293	305	312	58	0.15064935
LM 30/z	225	241	243	284	284	287	296	306	345	120	0.31168831
LM 31/z	178	184	201	221	235	230	234	239	263	85	0.22077922
LM 32/z	223	231	227	244	240	239	250	243	215	-8	-0.02077922
LM 33/z	216	207	205	223	229	230	233	241	245	29	0.07532468
LM 35/z	196	209	221	232	217	223	235	246	260	64	0.16623377
Total	5571	5813	6092	6587	6575	6583	6804	7037	5393	1356	3.52207792
Mean	**232**	**242**	**254**	**274**	**274**	**274**	**284**	**293**	**300**	**75**	**0.195671**

Table 2.7: Healthy buffalo calves

	10/10/00	4/12/00	15/1/01	3/4/01	8/5/01	7/6/01	13/7/01	7/8/01	30/10/01	Total gain	Average gain
LM 3/z	283	309	321	333	336	312	341	353	360	77	0.2
LM 11/z	244	256	280	311	289	305	346	328	338	94	0.24415584
LM 15/z	292	310	338	362	346	327	360	373	395	103	0.26753247
LM 18/z	174	180	184	205	207	208	216	225	235	61	0.15844156
LM 23/z	262	281	272	294	288	295	298	304	308	46	0.11948052
Total	1255	1336	1395	1505	1466	1447	1561	1583	1636	381	0.98961039
Mean	**251**	**267.2**	**279**	**301**	**293**	**289**	**312**	**317**	**327**	**76**	**0.19792208**

6 animals escaped from the bomas on the last day and thus could not be weighed.

Animals were euthanased from the helicopter and the carcasses could be retrieved from the veldt for necropsy purposes.

Table 3: Skin test results for the buffalo calves from the vaccination trial.

Nr.	Skin test at capture – 2000					13 July 2001					15 October 2001				
	0h B	72h B	0h A	72h A	Res.	0h B	72h B	0h A	72h A	Res.	0h B	72h B	0h A	72h A	Res.
Con.															
2	17,1	16,2	12,8	12,0	Neg.	22,5	∞	22,0	23,8	Pos.	19,3	∞	19,5	20,9	Pos.
4	14.0	13.8	13.3	13.3	Neg.	16.5	∞	15.9	18.2	Pos.	19.1	∞	16.9	20.2	Pos.
5	14.9	13.2	14.9	13.5	Neg.	12.5	18.8	12.0	13.0	Pos.	14.0	17.0	13.9	15.4	Neg.
7	12.3	11.1	13.2	12.8	Neg.	11.5	∞	11.9	15.6	Pos.	11.1	28.7	12.3	16.9	Pos.
8	10.0	11.0	11.4	11.1	Neg.	10.4	27.7	11.2	11.7	Pos.	11.4	20.0	12.3	12.7	Pos.
9	14.9	14.4	15.9	13.9	Neg.	13.6	∞	12.2	15.0	Pos.	13.9	28.2	19.5	20.9	Neg.
11	13.5	13.2	14.5	14.9	Neg.	14.6	15.8	13.9	15.7	Neg.	14.1	20.9	14.1	19.3	Equal
12	15.8	15.5	12.9	13.7	Neg.	14.7	24.9	15.0	19.9	Pos.	16.9	26.2	15.2	19.3	Pos.
15	16.5	16.4	17.0	17.0	Neg.	19.8	22.3	20.5	21.4	Sus.	17.5	20.1	19.0	23.7	Avian
20	19.6	19.8	18.9	18.9	Neg.	18.7	∞	17.3	19.0	Pos.	16.4	27.4	17.0	19.2	Pos.
24	18.0	16.1	15.1	15.0	Neg.	14.7	31.8	15.5	21.8	Pos.	15.0	23.0	15.5	19.5	Pos.
28	13.7	13.9	14.1	13.5	Neg.	13.1	∞	13.3	13.9	Pos.	15.6	∞	14.7	19.2	Pos.
31	10.9	10.4	11.9	11.3	Neg.	9.9	∞	9.7	15.1	Pos.	12.2	30.9	11.6	15.4	Pos.
33	15.1	16.1	15.1	14.4	Neg.	17.0	30.3	16.0	20.5	Pos.	14.1	∞	14.2	18.2	Pos.
Vacc															
1	14.9	14.5	16.1	16.0	Neg.	16.5	∞	16.9	24.6	Pos.	20.6	∞	20.9	27.9	Pos.
3	17.8	16.5	18.6	17.7	Neg.	17.6	30.0	17.7	23.2	Pos.	22.1	33.0	21.0	23.1	Pos.
10	17.6	17.7	17.8	17.1	Neg.	14.6	∞	14.7	16.4	Pos.	16.6	∞	15.5	18.1	Pos.
14	14.8	15.5	16.1	15.3	Neg.	15.8	∞	16.1	17.4	Pos.	15.0	∞	14.2	15.2	Pos.
16	14.2	12.8	12.6	12.0	Neg.	12.0	19.3	11.6	17.1	Pos.	13.3	19.1	12.8	16.5	Pos.
18	12.1	11.4	11.5	11.7	Neg.	10.0	16.5	10.9	18.8	Equal	10.3	16.7	10.8	15.0	Sus.
19	15.9	16.1	15.8	16.4	Neg.	15.9	∞	15.5	20.2	Pos.	14.2	25.9	18.5	23.2	Pos.
21	15.0	13.2	15.1	14.6	Neg.	14.3	∞	14.4	22.3	Pos.	16.0	∞	16.3	20.6	Pos.
23	20.9	20.4	21.1	19.7	Neg.	17.5	25.3	17.0	20.0	Pos.	18.4	20.5	20.0	17.8	Pos.
25	12.6	12.5	13.3	14.3	Neg.	14.4	21.7	13.0	19.4	Equal	12.4	19.7	14.5	19.0	Sus.
27	13.2	12.6	15.1	13.6	Neg.	13.7	19.1	12.9	18.9	Equal	13.7	19.3	12.3	18.1	Equal
29	17.4	16.8	15.6	15.9	Neg.	16.7	∞	16.3	18.3	Pos.	20.0	∞	18.8	20.0	Pos.
30	14.5	13.7	16.3	15.8	Neg.	15.0	27.0	14.1	21.0	Pos.	16.2	27.1	17.2	19.3	Pos.
32	12.7	13.0	13.1	12.2	Neg.	11.3	20.0	11.7	12.1	Pos.	10.6	21.4	9.6	12.0	Pos.
35	17.4	15.3	15.6	15.3	Neg.	12.3	15.0	12.9	17.4	Avian	14.7	17.4	15.0	18.0	Neg.

Table 4: Gamma-interferon results of the calves from the BCG vaccine study

Nr	10/10/2000	4/12/2000	15/1/2001	7/3/2001	3/4/2001	8/5/2001	7/6/2001	10/7/2001	7/8/2001	5/9/2001	12/10/2001	Result
Control												
LM 2/z	Neg.	Neg.	Avian	Neg.	Pos.	Invalid	Pos.	Pos.	MR	Pos.	Pos.	Pos.
LM 4/z	Neg.	Neg.	Avian	Neg.	Neg.	Pos.	Pos.	MR	Pos.	Pos.	Pos.	Pos.
LM 5/z	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.
LM 7/z	Neg.	Neg.	Neg.	Neg.	Neg.	MR	Pos.	MR	MR	Pos.	Pos.	Pos.
LM 8/z	Neg.	Avian	Avian	Avian	MR	Neg.	Pos.	Pos.	MR	Pos.	Pos.	Pos.
LM 9/z	Neg.	Equal	Avian	MR	Neg.	MR	Pos.	Pos.	Susp.	Pos.	Pos.	Pos.
LM 11/z	Neg.	Neg.	Neg.	Avian	Neg.	Avian	Avian	Neg.	Avian	Avian	Equal	Neg.
LM 12/z	Neg.	Avian	Neg.	Avian	Neg.	MR	Pos.	Neg.	Pos.	Pos.	Avian	Pos.
LM 15/z	Neg.	Neg.	Neg.	Susp.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
LM 20/z	Neg.	Neg.	Avian	Neg.	Neg.	Susp.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.
LM 24/z	Neg.	Neg.	Neg.	Neg.	Neg.	MR	Pos.	MR	MR	Neg.	Neg.	Pos.
LM 28/z	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Pos.	Pos.	Susp.	Pos.	Pos.	Pos.
LM 31/z	Neg.	Neg.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.
LM 33/z	Neg.	Neg.	Neg.	Neg.	MR	Equal	Pos.	Pos.	Pos.	Pos.	Avian	Pos.
Vaccine												
LM 1/z	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Susp.	Pos.	Pos.	Pos.	Pos.	Pos.
LM 3/z	Neg.	Neg.	Neg.	Neg.	Neg.	Avian	Pos.	Neg.	Avian	Neg.	Neg.	Neg.
LM 10/z	Neg.	Equal	Avian	Susp.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.
LM 14/z	Neg.	Neg.	Neg.	Equal	Neg.	Susp.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.
LM 16/z	Neg.	Avian	Avian	Avian	MR	Avian	Equal	Susp.	MR	Neg.	Neg.	Neg.
LM 18/z	Neg.	Avian	Avian	Avian	Neg.	Avian	Neg.	Neg.	Equal	Neg.	Neg.	Neg.
LM 19/z	Neg.	Neg.	Avian	Equal	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.
LM 21/z	Neg.	Equal	Neg.	Pos.	Neg.	Pos.	Pos.	Pos.	MR	Pos.	Pos.	Pos.
LM 23z	Neg.	Equal	Neg.	Neg.	Neg.	Neg.	Equal	Avian	Neg.	Neg.	Avian	Neg.
LM 25/z	Neg.	Neg.	Neg.	Neg.	Neg.	Invalid	Pos.	Invalid	Invalid	Pos.	Avian	Pos.
LM 27/z	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
LM 29/z	Neg.	Neg.	Neg.	Neg.	Neg.	MR	Pos.	Pos.	MR	Pos.	Suspect	Pos.
LM 30/z	Avian	Neg.	Avian	Pos.	Neg.	Pos.	Avian	Avian	MR	Neg.	Avian	Pos.
LM 32/z	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Suspect	Pos.
LM 35/z	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Table 5.1: Haematology results of the individual control animals from the BCG vaccine study.

LM33/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	30/10/2001	Total	Mean	Stdev
WBC	5.6	4.8	4.5	4.3	5.8	5.9	5.6	36.5	5.21	0.66
RBC	10.75	11.04	9.04	6.94	7.17	9.46	8.61	63.01	9	1.59
HGB g/dl	12.8	12.9	10.1	11.1	11.5	10.8	14.7	83.9	11.99	1.57
Hct %	37.9	38.3	31.3	29	30.2	33.6	36.2	236.5	33.79	3.77
MCV fl	35.2	34.7	34.7	41.8	42.1	35.5	42.1	266.1	38.01	3.74
MCH pg	11.9	11.7	11.2	16	16.1	11.4	17.1	95.4	13.63	2.63
MCHC g/dl	33.8	33.8	32.3	38.4	38.2	32.1	40.7	249.3	35.61	3.42
Platelets 10^3	1832	2218	1764	2110	2121	1650	2754	14449	2064	370
Lymphocyte %	97.5	71.1	79.7	75.3	88.5	82.2	73.1	567.4	81.06	9.35
Lymphocyte 10^3	5.5	3.4	3.5	3.2	5.2	4.8	4.1	29.7	4.24	9.93

LM 31/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	30/10/2001	Total	Mean	Stdev
WBC	10.7	9.7	9	8.3	10.7	7.8	8.2	64.4	9.2	1.19
RBC	11.21	11.17	10.39	6.51	6.67	10.75	8.45	65.15	9.31	2.08
HGB g/dl	11.8	11.6	10.8	12.9	11.7	12.2	15	86	12.29	1.36
Hct %	33.6	34.3	32.5	27	27.2	37.7	35.6	227.9	32.56	4.07
MCV fl	30	30.8	31.3	41.5	41.5	35.1	42.2	252.4	36.06	5.55
MCH pg	10.6	10.4	10.4	19.8	17.5	11.3	17.8	97.8	13.97	4.19
MCHC g/dl	35.3	33.8	33.3	47.8	42.2	32.3	42.2	266.9	38.13	5.93
Platelets 10^3	4987	5070	4616	3566	3286	1699	3407	26631	3804	1195
Lymphocyte %	95.2	55.2	74.3	67.8	78.6	75.1	73.4	519.6	74.23	12
Lymphocyte 10^3	10.2	5.3	6.7	5.6	8.4	5.9	6	48.1	6.87	1.79

LM 28/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	2/11/2001	Total	Mean	Stdev
WBC	8.8	6.8	6.8	7	7.2	7.5		44.1	7.35	0.76
RBC	10.3	7.7	8.65	7.24	8.49	7.03		49.41	8.24	1.2
HGB g/dl	13.9	10.7	12.4	11.5	12.8	10		71.3	11.88	1.43
Hct %	39.5	31	35.2	32.7	38.8	29.4		206.6	34.43	4.13
MCV fl	38.4	40.2	41.9	45.2	45.7	41.8		253.2	42.2	2.83
MCH pg	13.5	13.9	14.3	15.9	15.1	14.2		86.9	14.48	0.87
MCHC g/dl	35.3	34.7	34.1	35.2	33.1	34		206.4	34.4	0.83
Platelets 10^3	896	434	475	651	853	517		3826	638	198
Lymphocyte %	94.1	63.5	70.7	69.1	79.8	77.7		454.9	75.82	10.74
Lymphocyte 10^3	8.3	4.3	4.8	4.8	5.8	5.8		33.8	5.63	1.44

LM 24/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	1/11/2001	Total	Mean	Stdev
WBC	8.1	8.7	6.1	9.4	9.4	10.6	7	59.3	8.47	1.54
RBC	7.64	7.82	7.31	7.34	7.13	7.91	10.04	55.19	7.88	0.99
HGB g/dl	10	10.2	9.1	10.8	10.6	9.6	15.6	75.9	10.84	2.18
Hct %	28.4	29.8	28.4	31.6	30.3	29.4	43	220.9	31.56	5.17
MCV fl	37.1	38.1	38.9	43.1	42.5	37.2	42.8	279.7	39.96	2.73
MCH pg	13.1	13	12.4	14.7	14.8	12.2	15.5	95.7	13.67	1.31
MCHC g/dl	35.3	34.1	32	34.1	34.9	32.8	36.3	239.5	34.21	1.47
Platelets 10^3	969	950	1003	1241	1350	1098	2031	8642	1235	381
Lymphocyte %	93.4	44.6	57.4	55.9	69.6	64.9	64.4	450.2	64.31	15.17
Lymphocyte 10^3	7.5	3.9	3.5	5.2	6.6	6.9	4.5	38.1	5.44	1.57

LM 20/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	2/11/2001	Total	Mean	Stdev
WBC	5.6	13	8.9	12.4	12.3	15.2		67.4	11.23	3.42
RBC	8.59	9.87	9.8	10.28	9.92	11.2		59.66	9.94	0.84
HGB g/dl	11.7	15.4	13.3	16.4	15.4	15.2		87.4	14.57	1.73
Hct %	32.7	43.3	40.1	46.2	44.3	45.7		252.3	42.05	5.07
MCV fl	38	43.9	40.9	44.9	44.6	40.8		253.1	42.18	2.73
MCH pg	13.6	15.7	13.6	16	15.4	13.5		87.8	14.63	1.18
MCHC g/dl	35.7	35.7	33.3	35.5	34.6	33.2		208	34.67	1.17
Platelets 10^3	868	1358	805	1252	1231	784		6298	1050	258
Lymphocyte %	97	51.6	63	59.8	73	67.4		411.8	68.63	15.65
Lymphocyte 10^3	5.5	6.7	5.6	7.4	9	10.3		44.5	7.42	1.91

LM 15/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	9.6	8.8	6.8	8.9	9.3	10.6	6.1	60.1	8.59	1.59
RBC	9.59	7.23	10.14	7.97	8.86	9.67	9.38	62.84	8.98	1.04
HGB g/dl	11.6	13.4	13.1	13.3	14.3	12.2	16.7	94.6	13.51	1.65
Hct %	32.6	30.5	37	33.8	37.8	35.5	40.2	247.4	35.34	3.31
MCV fl	34	42.2	36.5	42.3	42.7	36.7	42.8	277.2	39.6	3.73
MCH pg	12.1	18.6	12.9	16.6	16.2	12.7	17.8	106.9	15.27	2.66
MCHC g/dl	45.6	44	35.4	39.3	37.9	34.5	41.5	278.2	39.74	4.19
Platelets 10^3	2294	2437	1293	1606	1926	1302	2294	13152	1879	485
Lymphocyte %	95.9	72.4	58.9	63.1	67.5	66.7	62.8	487.3	69.61	12.35
Lymphocyte 10^3	9.2	6.4	4	5.6	6.3	7.1	3.8	42.4	6.06	1.86

LM 12/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	2/11/2001	Total	Mean	Stdev
WBC	6.6	7.8	5.4	6.1	7.1	***		33	6.6	0.92
RBC	8.43	6.03	6.36	6.11	6.45	8.59		41.97	7	1.19
HGB g/dl	10.8	9.5	9.6	9.8	9.9	11.4		61	10.17	0.76
Hct %	31.3	25.5	27.7	26.3	28.2	33.1		172.1	28.68	2.95
MCV fl	37.2	42.4	43.5	43	43.7	38.6		248.4	41.4	2.78
MCH pg	12.8	15.7	15.2	16	15.4	13.3		88.4	14.7	1.34
MCHC g/dl	34.4	37	34.9	37.1	35.1	34.4		212.9	35.48	1.25
Platelets 10^3	1177	1283	1076	1074	1051	841		6502	1084	147
Lymphocyte %	96	62.5	65.9	69.9	73.4	***		367.7	73.54	13.21
Lymphocyte 10^3	6.3	4.9	3.6	4.3	5.2	***		24.3	4.86	1.01

LM 11/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	01/11/2001	Total	Mean	Stdev
WBC	7.9	9.1	5.8	4.6	6.8	10.7	6.5	51.4	7.34	2.07
RBC	9.92	6.17	8	5.37	7.08	9.34	8.96	54.84	7.83	1.7
HGB g/dl	11.8	11	9.6	8.7	11	11.4	16.1	79.6	11.37	2.35
Hct %	34.5	25.6	28	22.2	29.9	33.6	38.2	212	30.29	5.54
MCV fl	34.7	41.5	35	41.4	42.2	35.9	42.6	273.3	39.04	3.64
MCH pg	11.9	17.9	12	16.3	15.5	12.2	17.9	103.7	14.81	2.74
MCHC g/dl	34.4	43.1	34.1	39.4	36.7	34	42.1	263.8	37.69	3.87
Platelets 10^3	1882	2310	1548	1644	1767	1511	2731	13393	1913	450
Lymphocyte %	95.2	44.3	60.5	66.3	70.7	71.3	66.6	474.9	67.84	15.17
Lymphocyte 10^3	7.5	4	3.5	3.1	4.8	7.7	4.2	34.8	4.97	1.87

LM 9/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	1/11/2001	Total	Mean	Stdev
WBC	7.6	7	5.7	8.3	12.8	10	5.9	57.3	8.19	2.51
RBC	11.36	5.17	9.85	6.35	8.03	11.56	8.83	61.15	8.74	2.42
HGB g/dl	12.1	10.7	10.3	11.6	14	12.7	16.3	87.7	12.53	2.07
Hct %	35.5	21	31.7	26.4	34	39	37.2	224.8	32.11	6.39
MCV fl	31.2	40.6	32.2	41.7	42.4	33.8	42.2	264.1	37.73	5.07
MCH pg	10.7	20.6	10.5	18.2	17.5	11	18.4	106.9	15.27	4.35
MCHC g/dl	34.2	50.7	32.5	43.8	41.3	32.6	43.7	278.8	39.83	6.94
Platelets 10^3	4018	3658	3768	3223	3236	2842	3439	24184	3455	395
Lymphocyte %	93	70.6	61.5	60.8	73.5	69.1	78.3	506.8	72.4	11.01
Lymphocyte 10^3	7.1	4.9	3.5	5.1	9.4	6.9	4.6	41.5	5.93	1.99

LM 8/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	1/11/2001	Total	Mean	Stdev
WBC	7.4	6.8	5	5	8.8	8.9	6.1	48	6.86	1.62
RBC	9.74	6.54	8.19	9.01	6.74	9.4	10.57	60.19	8.6	1.52
HGB g/dl	12.2	10.9	10	14.9	10.8	12.5	18.2	89.5	12.79	2.87
Hct %	34.3	27.2	29.7	39	29	35.7	46.8	241.7	34.53	6.83
MCV fl	35.2	41.6	36.3	43.2	43	38	44.3	281.6	40.23	3.67
MCH pg	12.5	16.6	12.2	16.5	16.1	13.2	17.2	104.3	14.9	2.17
MCHC g/dl	35.6	39.9	33.6	38.1	37.4	34.9	38.9	258.4	36.91	2.28
Platelets 10^3	1823	2082	1229	1885	1776	1207	2165	12167	1738	381
Lymphocyte %	92.7	72.7	62.6	71.7	71.7	69.6	65.7	506.7	72.39	9.68
Lymphocyte 10^3	6.8	4.9	3.1	3.6	6.3	6.2	4	34.9	4.99	1.47

LM 7/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	1/11/2001	Total	Mean	Stdev
WBC	7.1	7.9	7.7	4.8	8.5	***	3.3	39.3	6.55	2.04
RBC	8.52	5.73	8.69	5.63	6	10.03	8.88	53.48	7.64	1.8
HGB g/dl	9.7	10.6	9.7	9.6	10	11.7	15.4	76.7	10.96	2.1
Hct %	29	23.3	29.9	22.9	25.1	35.2	37.7	203.1	29.01	5.77
MCV fl	34	40.6	34.4	40.7	41.8	35.2	42.4	269.1	38.44	3.73
MCH pg	11.4	18.4	11.2	17.1	16.8	11.6	17.3	103.8	14.83	3.25
MCHC g/dl	33.4	45.4	32.5	42	40.1	33.1	40.9	267.4	38.2	5.14
Platelets 10^3	2116	2799	2071	2490	2364	2250	2953	17043	2435	336
Lymphocyte %	93	63.7	57.3	67.6	75	***	75.7	432.3	72.05	12.39
Lymphocyte 10^3	6.7	5	4.4	3.2	6.4	***	2.5	28.2	4.7	1.68

LM 5/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	1/11/2001	Total	Mean	Stdev
WBC	6.9	5.1	6.3	9.8	10.1	8.4	7.5	54.1	7.73	1.83
RBC	9.32	6.05	8.98	7.94	6.6	9.41	8.37	56.67	8.1	1.33
HGB g/dl	10.6	11.2	10.1	13	10.7	10.7	16	82.3	11.76	2.09
Hct %	30.8	24.7	30.8	33.5	27.2	32.1	35.1	214.2	30.6	3.59
MCV fl	33.1	40.9	34.3	42.1	41.2	34.1	41.9	267.6	38.23	4.15
MCH pg	11.4	18.5	11.3	17.4	16.2	11.4	19.1	105.3	15.04	3.56
MCHC g/dl	34.5	45.3	32.9	41.4	39.4	33.4	45.7	272.6	38.94	5.47
Platelets 10^3	2260	2736	1838	2539	2057	1981	3129	16540	2363	462
Lymphocyte %	92.7	74.7	73.5	61.3	69.1	71.4	74.4	517.1	73.87	9.51
Lymphocyte 10^3	6.4	3.8	4.6	6	7	6	5.6	39.4	5.63	1.09

LM 4/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	11.4	11.5	8.2	9.2	11.08	14.3	6.7	72.38	10.34	2.51
RBC	8.86	8.44	8.75	7.92	9.2	8.78	10.64	62.59	8.94	0.85
HGB g/dl	11.7	14	12.2	12.8	13.7	12.6	17.6	94.6	13.51	1.97
Hct %	33.7	36.9	36.1	36	42	36.6	47.6	268.9	38.41	4.77
MCV fl	38	43.8	41.3	45.4	46.2	41.6	44.8	301.1	43.01	2.88
MCH pg	13.2	16.6	13.9	16.1	15.1	14.3	16.5	105.7	15.1	1.35
MCHC g/dl	34.9	37.8	33.8	35.5	32.7	34.5	37	246.2	35.17	1.77
Platelets 10^3	875	1324	591	717	910	634	945	5996	857	248
Lymphocyte %	94.5	72.6	75.8	61.2	78.5	68.5	50.6	501.7	71.67	13.84
Lymphocyte 10^3	10.8	8.2	6.2	5.6	9.2	9.8	3.4	53.2	7.6	2.63

LM 2/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	10.5	11.7	7.8	7.1	9.8	***	8.5	55.4	9.23	1.74
RBC	10.11	7.2	8.24	7.11	6.59	10.6	7.13	56.98	8.14	1.6
HGB g/dl	11.5	14.2	9.6	13.3	12.1	12.1	15	87.8	12.54	1.8
Hct %	34.1	30.5	28.4	30	27.6	35.3	30.2	216.1	30.87	2.83
MCV fl	33.8	42.4	34.5	42.2	41.9	33.3	42.4	270.5	38.64	4.48
MCH pg	11.4	19.7	11.6	18.7	18.4	11.4	21	112.2	16.03	4.35
MCHC g/dl	33.8	46.6	33.8	44.4	43.9	34.2	49.5	286.2	40.89	6.75
Platelets 10^3	2718	3215	2141	2894	2954	3573	3881	21376	3054	572
Lymphocyte %	87.8	70.4	61.6	58.6	72.9	***	59.3	410.6	68.43	11.19
Lymphocyte 10^3	9.2	8.2	4.8	4.2	7.1	***	5.1	38.6	6.43	2.03

Table 5.2: Haematology results of the individual vaccinated animals from the BCG vaccine study.

LM 35/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	29/10/2001	Total	Mean	Stdev
WBC	5.8	6	7.1	8.3	7.2	11.7	7.9	54	7.71	1.98
RBC	5.1	5.01	4.84	5.99	6.16	8.34	10.85	46.29	6.61	2.22
HGB g/dl	6.4	7.9	7.7	9.3	9.2	10.1	13.7	64.3	9.19	2.34
Hct %	18.6	22.4	22.8	27.6	27.3	32	41.4	192.1	27.44	7.54
MCV fl	36.4	44.8	47	46.1	44.4	38.3	38.2	295.2	42.17	4.37
MCH pg	12.7	15.8	15.9	15.5	14.9	12.1	12.6	99.5	14.21	1.68
MCHC g/dl	34.8	35.3	33.8	33.6	33.7	31.7	33.1	236	33.71	1.17
PLT 10^3	814	971	590	731	1035	1079	1346	6566	938	250
Lymph %	97.4	66.6	75.1	71.7	77.6	71.7	69.8	529.9	75.7	10.2
Lym 10^3	5.6	4	5.4	6	5.6	8.4	5.5	40.5	5.79	1.32

LM 32/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	10.8	11.2	9.6	14.5	12.7	9.5	6.1	74.4	10.63	2.66
RBC	8.03	8.99	6.29	6.28	5.82	7.34	5.13	47.88	6.84	1.35
HGB g/dl	10	11.3	7.5	9.9	9.2	8.1	9.2	65.2	9.31	1.26
Hct %	29.6	33.9	22.8	26.3	24.7	25.4	21	183.7	26.24	4.33
MCV fl	36.8	37.7	36.2	41.9	42.5	34.6	41	270.7	38.67	3.1
MCH pg	12.5	12.5	11.9	15.8	15.9	11	18.1	97.7	13.96	2.63
MCHC g/dl	33.8	33.3	33	37.8	37.4	31.9	44.1	251.3	35.9	4.26
PLT 10^3	1217	1394	1332	2139	2063	2327	2911	13383	1912	624
Lymph %	92.6	53.3	64.9	63.2	63.7	61.8	35.3	434.8	62.11	17.02
Lym 10^3	10	6	6.2	9.2	8.1	5.8	2.1	47.4	6.77	2.64

LM30/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	7	4.6	6.2	6.6	7.4	7.9	3.2	42.9	6.13	1.67
RBC	10.84	8.18	11.36	7.5	7.09	8.15	7.71	60.83	8.69	1.7
HGB g/dl	12.3	9.2	12.7	12.9	11.8	9	14.5	82.4	11.77	2.01
Hct %	35.3	26.7	38.6	31.1	29.5	27.2	32	220.4	31.49	4.3
MCV fl	32.6	32.7	34	41.4	41.6	33.4	41.5	257.2	36.74	4.47
MCH pg	11.3	11.3	11.2	17.2	16.7	11	18.8	97.5	13.5	3.46
MCHC g/dl	34.7	34.6	32.9	41.4	40.1	33.1	45.2	262	37.43	4.8
PLT 10^3	3169	2400	2394	2660	2487	2514	2816	18440	2634	279
Lymph %	95.5	64.1	75.1	70.8	73.9	79.7	76.8	535.9	76.56	9.72
Lym 10^3	6.7	3	4.7	4.7	5.5	6.3	2.4	33.3	4.76	1.6

LM 29/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	1/11/2001	Total	Mean	Stdev
WBC	14.3	14.9	15.5	13.8	18.5	16	10.3	103.3	14.76	2.49
RBC	8.69	8.68	7.75	7.37	8.74	8.25	9.77	59.25	8.46	0.78
HGB g/dl	12	12.3	10.1	10.9	12.5	10.9	15.2	83.9	11.99	1.66
Hct %	34.6	35.6	32.2	32.7	39.3	34.1	43.9	252.4	36.06	4.17
MCV fl	39.8	41	41.5	44.3	45	41.3	45	297.9	42.56	2.15
MCH pg	13.9	14.2	13.1	14.9	14.3	13.2	15.5	99.1	14.16	0.86
MCHC g/dl	34.8	34.6	31.5	33.5	31.8	32	34.5	232.7	33.24	1.45
PLT 10^3	667	647	542	584	852	604	1093	4989	712	195
Lymph %	95.2	61.8	60.7	66.7	68.8	77.9	71.4	502.5	71.79	11.85
Lym 10^3	13.6	9.2	9.4	9.2	12.7	12.4	7.3	73.8	10.54	2.34

LM 27/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	29/10/2001	Total	Mean	Stdev
WBC	6.3	6.1	4.5	5.5	7.2	7.5	6.1	43.2	6.17	1.01
RBC	10.62	11.25	9.63	9.09	10.55	9.15	12.95	73.24	10.46	1.36
HGB g/dl	14	15.3	13	13.3	15.3	12	17.9	100.8	14.4	1.96
Hct %	41.8	47.2	40.7	40.8	48.4	37.8	54.4	311.1	44.44	5.79
MCV fl	39.4	42	42.3	44.9	45.9	41.3	42	297.8	42.54	2.2
MCH pg	13.2	13.5	13.5	14.6	14.5	13.1	13.9	96.3	13.76	0.6
MCHC g/dl	33.5	32.3	31.8	32.7	31.6	31.7	33	226.6	32.37	0.73
PLT 10^3	879	558	368	706	940	531	655	4637	662	200
Lymph %	95	57.4	53.3	66.4	62.8	59.7	50	444.6	63.51	14.94
Lym 10^3	6	3.5	2.3	3.6	4.5	4.5	3	27.4	3.91	1.21

LM 25/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	30/10/2001	Total	Mean	Stdev
WBC	5.7	6.6	5.5	7.5	10.1	8.5	4.1	48	6.86	2.02
RBC	9.01	8.83	7.16	8.44	8.25	8.56	10.69	60.94	8.71	1.06
HGB g/dl	11.2	12.1	9.2	13.2	13	11.5	17.2	87.4	12.49	2.47
Hct %	34	34.3	27.6	38.4	37.2	34	47.5	253	36.14	6.07
MCV fl	37.8	38.8	38.6	43.5	45.1	39.7	44.5	288	41.14	3.1
MCH pg	12.5	13.6	12.8	15.7	15.7	13.4	16.1	99.8	14.26	1.52
MCHC g/dl	33	35.2	33.2	34.3	34.9	33.8	36.2	240.6	34.37	1.15
PLT 10^3	1169	1115	905	1313	1299	826	1489	8116	1159	234
Lymph %	97	56.7	73.5	80	77.1	76.2	75.1	535.6	76.51	11.8
Lym 10^3	5.5	3.7	4.1	6	7.8	6.5	3.1	36.7	5.24	1.69

LM 23/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	30/10/2001	Total	Mean	Stdev
WBC	7.3	6.7	5	6.2	3.9	7.9	7.2	44.2	6.31	1.41
RBC	12.53	11.74	10.14	8.4	8.93	11.25	9.93	72.92	10.42	1.5
HGB g/dl	15.1	14.5	11.9	14.6	15.4	13.4	17.8	102.7	14.67	1.82
Hct %	42.7	50.9	35.4	35.5	38	40.1	42.8	285.4	40.77	5.4
MCV fl	34.1	34.8	34.9	42.3	42.5	35.6	43.1	267.3	38.19	4.19
MCH pg	12.1	12.4	11.7	17.3	17.3	11.9	17.9	100.6	14.37	2.94
MCHC g/dl	35.4	35.6	33.6	41	40.7	33.3	41.5	261.1	37.3	3.63
PLT 10^3	2965	2593	2238	2727	2842	2320	3065	18750	2679	314
Lymph %	97.6	49	67.2	72.6	72.1	73.9	67.5	499.9	71.41	14.31
Lym 10^3	7.2	3.3	3.3	4.5	2.8	5.9	4.9	31.9	4.56	1.59

LM 21/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	5.6	6.5	5.6	6	6.7	7	3.7	41.1	5.87	1.1
RBC	8.65	6.97	8.48	7.9	8.51	9.3	11.07	60.88	8.7	1.27
HGB g/dl	11.7	11.4	11.7	12.4	12.6	12.9	18.1	90.8	12.97	2.33
Hct %	32.8	30.2	34.3	34.6	37.5	37.5	48.9	255.8	36.54	6.02
MCV fl	37.9	43.3	40.4	43.8	44.2	40.3	44.2	294.1	42.01	2.48
MCH pg	13.5	16.3	13.9	15.7	14.9	13.9	16.4	104.6	14.94	1.21
MCHC g/dl	35.7	37.8	34.3	35.9	33.7	34.3	37.1	248.8	35.54	1.54
PLT 10^3	913	1150	730	908	1121	727	1172	6721	960	191
Lymph %	97.3	53.1	69.1	63.3	77.4	70.3	92.4	522.9	74.7	15.7
Lym 10^3	5.4	3.5	3.9	3.8	5.2	4.9	3.4	30.1	4.3	0.84

LM 19/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	7.5	8.6	8.5	8	9.2	9.8		51.6	8.6	0.82
RBC	9.13	5.68	8.8	6.8	8.59	9.66		48.66	8.11	1.53
HGB g/dl	11.3	10	10.6	10.8	13.3	11.4		67.4	11.23	1.13
Hct %	32.6	23.4	32	28.4	36.5	34.8		187.7	31.28	4.74
MCV fl	35.6	41.2	36.3	41.8	42.5	36		233.4	38.9	3.25
MCH pg	12.4	17.6	12.1	15.8	15.5	11.8		85.2	14.2	2.42
MCHC g/dl	34.6	42.7	33.2	37.9	36.5	32.8		217.7	36.28	3.7
PLT 10^3	1486	1803	1253	1474	1729	1534		9279	1547	197
Lymph %	93.1	52.1	62.6	60.1	71	70.5		409.4	68.23	14.07
Lym 10^3	7	4.5	5.3	4.8	6.5	6.9		35	5.83	1.1

LM 18/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	29/10/2001	Total	Mean	Stdev
WBC	7.1	6.8	5.8	6.4	7.9	9.3	5.5	48.8	6.97	1.3
RBC	10.17	8.24	8.62	8.33	8.9	10.08	14.06	68.4	9.77	2.05
HGB g/dl	12.1	12.1	10.3	12	12.7	12.4	18.8	90.4	12.91	2.71
Hct %	37.5	35	33.1	36.3	39.7	40.2	58.1	279.9	39.99	8.37
MCV fl	36.9	42.4	38.5	43.6	44.6	39.8	41.4	287.2	41.03	2.78
MCH pg	11.9	14.7	12	14.4	14.2	12.3	13.3	92.8	13.26	1.2
MCHC g/dl	32.4	34.7	31.3	33.1	31.9	30.9	32.3	226.6	32.37	1.26
PLT 10^3	1291	1510	833	1245	1075	758	645	7357	1051	318
Lymph %	97.2	61.2	64.1	66.5	69.3	62.2	80	500.5	71.5	12.97
Lym 10^3	7	4.2	3.7	4.3	5.5	5.8	4.4	34.9	4.99	1.16

LM 16/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	29/10/2001	Total	Mean	Stdev
WBC	9.1	9	7.1	5	9.7	7.8	8.9	56.6	8.09	1.62
RBC	11.37	6.17	9.44	6.21	8.37	9.33	13.17	64.06	9.15	2.56
HGB g/dl	13.2	11.9	10.7	10.4	13.6	10.9	16	86.7	12.39	2.02
Hct %	37.9	25.4	31.7	25.7	35.5	32.8	47.9	236.9	33.84	7.75
MCV fl	33.3	41.2	33.6	41.4	42.4	35.1	36.4	263.4	37.63	3.93
MCH pg	11.7	19.4	11.3	16.8	16.2	11.7	12.2	99.3	14.19	3.23
MCHC g/dl	35	47.1	33.7	40.4	38.2	33.2	33.5	261.1	37.3	5.1
PLT 10^3	2745	2869	2290	2269	2361	1638	2115	16287	2327	407
Lymph %	95.2	68.6	70.2	60.8	74.6	67.5	69.9	506.8	72.4	10.87
Lym 10^3	8.6	6.2	5	3.1	7.2	5.3	6.3	41.7	5.96	1.74

LM 14/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	2/11/2001	Total	Mean	Stdev
WBC	8.7	8.8	6.8	5.8	12.7	9.9		52.7	8.78	2.43
RBC	11.25	9.18	8.52	8.85	9.38	10.43		57.61	9.6	1.04
HGB g/dl	15.2	15.8	10.8	14.3	14.4	13.2		83.7	13.95	1.78
Hct %	41.9	39.6	31.8	38.1	41	38.1		230.5	38.42	3.58
MCV fl	37.2	43.1	37.3	43.1	43.8	36.5		241	40.17	3.5
MCH pg	13.5	17.2	12.7	16.1	15.4	12.7		87.6	14.6	1.9
MCHC g/dl	36.3	39.9	34	37.4	35.2	34.8		217.6	36.27	2.14
PLT 10^3	1434	2126	1194	1584	1823	1918		10079	1680	341
Lymph %	95.6	54.7	56.6	39.6	60.7	60.5		367.7	61.28	18.52
Lym 10^3	8.3	4.8	3.8	2.3	7.7	6		32.9	5.48	2.3

LM 10/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	2/11/2001	Total	Mean	Stdev
WBC	8.9	9.8	7.9	6.2	9	11		52.8	8.8	1.64
RBC	11.28	8.4	9.02	9.22	10.48	10.76		59.16	9.86	1.14
HGB g/dl	14.5	13.2	12	14.3	16	14.4		84.4	14.07	1.35
Hct %	41.6	35.8	36.5	40.7	47	44.2		245.8	40.97	4.34
MCV fl	36.9	42.6	40.4	44.1	44.9	41.1		250	41.67	2.9
MCH pg	12.8	15.7	13.2	15.6	15.3	13.4		86	14.33	1.34
MCHC g/dl	34.8	36.8	32.8	35.3	34.1	32.7		206.5	34.42	1.57
PLT 10^3	1229	1444	820	867	1139	786		6285	1048	265
Lymph %	91.5	65.5	63.3	69.3	70.9	69.4		429.9	71.65	10.13
Lym 10^3	8.2	6.4	5	4.3	6.7	7.6		38.2	6.37	1.49

LM 3/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	29/10/2001	Total	Mean	Stdev
WBC	5.9	7.4	4.9	5	7.8	10	7.6	48.6	6.94	1.82
RBC	7.34	6.88	6.74	6.77	7.35	7.14	11.71	53.93	7.7	1.78
HGB g/dl	9.6	10.9	9.7	10.3	11.1	9.9	17	78.5	11.21	2.62
Hct %	28.4	30.1	28.5	30	32.7	29.6	49.7	229	32.71	7.62
MCV fl	38.7	43.8	42.3	44.4	44.6	41.4	42.4	297.6	42.51	2.06
MCH pg	13.1	15.8	14.4	15.3	15.1	13.9	14.5	102.1	14.59	0.91
MCHC g/dl	33.9	36.2	34.1	34.4	33.9	33.5	34.3	240.3	34.33	0.88
PLT 10^3	623	905	401	547	606	510	688	4280	611	159
Lymph %	95.5	70.3	69.4	66.3	75.7	74.7	73.4	525.3	75.04	9.6
Lym 10^3	5.6	5.2	3.4	3.3	5.9	7.5	5.6	36.5	5.21	1.47

LM 1/z	03/04/2001	08/05/2001	07/06/2001	13/07/2001	07/08/2001	12/10/2001	31/10/2001	Total	Mean	Stdev
WBC	9.7	9.3	8.4	10.6	10.3	10.5	3.6	62.4	8.91	2.47
RBC	7.65	7.58	8.44	8.26	7.59	7.54	10.81	57.87	8.27	1.18
HGB g/dl	11.6	11.8	11.1	12.8	10.9	9.7	10.8	78.7	11.24	0.96
Hct %	32.7	32.9	34.9	36.9	33.6	29.6	47.7	248.3	35.47	5.83
MCV fl	42.7	43.4	41.4	44.7	44.3	39.3	44.1	299.9	42.84	1.92
MCH pg	15.2	15.6	13.2	15.4	14.3	12.9	10	96.6	13.8	1.98
MCHC g/dl	35.6	35.9	31.8	34.5	32.4	32.8	22.6	225.6	32.23	4.53
PLT 10^3	1328	1100	533	802	853	599	1374	6589	941	335
Lymph %	90.8	66.5	67.5	57.7	82.8	72.1	65.4	502.8	71.83	11.32
Lym 10^3	8.9	6.2	5.6	6.1	8.6	7.6	2.3	45.3	6.47	2.24

Table 5.3: The mean haematology values of diseased and healthy animals of the BCG study.

Diseased	WBC 10 ^ 3	RBC 10 ^ 6	HBG g/dl	Hct %	MCV fl	MCH pg	MCHC g/d	PLT 10 ^ 3	Lymphocyte %	Lymphocyte 10 ^ 3
LM 35/z	7.71	6.61	9.19	27.44	42.17	14.21	33.71	938	75.7	5.79
LM 33/z	5.21	9	11.99	33.79	38.01	13.63	35.61	2064	81.06	4.24
LM 32/z	10.63	6.84	9.31	26.24	38.67	13.96	35.9	1912	62.11	6.77
LM 31/z	9.2	9.31	12.29	32.56	36.06	13.97	38.13	3804	74.23	6.87
LM 30/z	6.13	8.69	11.77	31.49	36.74	13.5	37.43	2634	67.56	4.76
LM 29/z	14.76	8.46	11.9	36.06	42.56	14.16	33.24	712	71.97	10.54
LM 28/z	7.35	8.24	11.88	34.43	42.2	14.48	34.4	638	75.82	5.63
LM 27/z	6.17	10.46	14.4	44.44	42.54	13.76	32.37	662	63.51	3.91
LM 25/z	6.86	8.71	12.49	36.14	41.14	14.26	34.37	1159	76.51	5.24
LM 24/z	8.47	7.88	10.84	31.56	39.96	13.67	34.21	1235	64.31	5.44
LM 21/z	5.87	8.7	12.97	36.54	42.01	14.94	35.54	960	74.7	4.3
LM 20/z	11.23	9.94	14.57	42.05	42.18	14.63	34.67	1050	68.63	7.42
LM 19/z	8.6	8.11	11.23	31.28	38.9	14.2	36.28	1547	68.23	5.83
LM 16/z	8.09	9.15	12.39	33.84	37.63	14.19	37.3	2327	72.4	5.96
LM 14/z	8.78	9.6	13.95	38.42	40.17	14.6	36.27	1680	61.28	5.48
LM 12/z	6.6	7	10.17	28.68	41.4	14.7	35.48	1084	73.54	4.86
LM 10/z	8.8	9.86	14.07	40.97	41.67	14.33	34.42	1048	71.65	6.37
LM 9/z	8.19	8.74	12.53	32.11	37.73	15.27	39.83	3455	72.4	5.93
LM 8/z	6.86	8.6	12.79	34.53	40.23	14.9	36.91	1738	72.39	4.99
LM 7/z	6.55	7.64	10.96	29.01	38.44	14.83	38.2	2435	72.05	4.7
LM 5/z	7.73	8.1	11.76	30.6	38.23	15.04	38.94	2363	73.87	5.63
LM 4/z	10.34	8.94	13.51	38.41	43.01	15.1	35.17	857	71.67	7.6
LM 2/z	9.23	8.14	12.54	30.87	38.64	16.03	40.89	3054	68.43	6.43
LM 1/z	8.91	8.27	11.24	35.47	42.84	13.8	32.23	941	71.83	6.47
Mean	**8.26**	**8.54**	**12.11**	**34.04**	**40.13**	**14.42**	**35.9**	**1679**	**71.08**	**5.88**
Stdev	2.0304706	0.933396	1.40308	4.4833	2.1414	0.5952	2.1920899	892.327	4.70419	1.356502

Healthy	WBC 10 ^ 3	RBC 10 ^ 6	HBG g/dl	HCT %	MCV fl	MCH pg	MCHC g/d	PL 10^3	Lymphocyte %	Lymphocyte 10 ^ 3
LM 23/z	6.31	10.42	14.67	40.77	38.19	14.37	37.3	2679	71.41	4.56
LM 18/z	6.97	9.77	12.91	39.99	41.03	13.26	32.37	1051	71.5	4.99
LM 15/z	8.59	8.98	13.51	35.34	39.6	15.27	39.74	1879	69.61	6.06
LM 11/z	7.34	7.83	11.37	30.29	39.04	14.81	37.69	1913	67.84	4.97
LM 3/z	6.94	7.7	11.21	32.71	42.51	14.59	34.33	611	75.04	5.21
Mean	**7.23**	**8.94**	**12.73**	**35.82**	**40.07**	**14.46**	**36.29**	**1627**	**71.08**	**5.16**
Stdev	0.8455472	1.18855	1.308765	4.538	1.5291	0.74893	2.6111504	723.331	2.675136	0.49749

Table 6: Grading of the macroscopic tuberculous lesions in experimental animals from the BCG vaccine study

Nr.	Tonsil	Head Inn.	Thorax Inn.	Lung	Other	Grading
Controls						**34**
LM 2/z	-	+	+	+	+	5
LM 4/z	-	+	+	-	-	3
LM 5/z	-	-	-	-	-	0
LM 7/z	+	+	+	-	+	5
LM 8/z	+	+	-	-	-	3
LM 9/z	+	+	-	-	-	3
LM 11/z	-	-	-	-	-	0
LM 12/z	-	+	-	-	-	1
LM 15/z	-	-	-	-	-	0
LM 20/z	-	+	-	-	-	2
LM 24/z	-	-	-	-	-	0
LM 28/z	+	+	-	-	-	3
LM 31/z	+	+	+	+	-	4
LM 33/z	+	+	+	+	-	5
	6/14	10/14	5/14	3/13	2/14	
Vaccine group						**21**
LM 1/z	-	+	-	-	-	2
LM 3/z	-	-	-	-	-	0
LM 10/z	+	+	+	-	-	3
LM 14/z	+	+	-	-	? Atypical	4
LM 16/z	-	-	-	-	-	0
LM 18/z	-	-	-	-	-	0
LM 19/z	-	+	+	+	-	5
LM 21/z	-	+	-	-	-	1
LM 23/z	-	-	-	-	-	0
LM 25/z	-	-	-	-	-	0
LM 27/z	-	-	-	-	-	0
LM 29/z	-	+	-	-	-	1
LM 30/z	-	-	-	-	-	0
LM 32/z	-	+	+	+	+	5
LM 35/z	-	-	-	-	-	0
	2/15	7/15	3/15	2/15	1/15	

Table 7: *Mycobacterium bovis* culture results of the BCG vaccine study

Number.	Left Tonsil	Right Tonsil	Left Retro In.	Right Retro In.	Head Inn.	Thorax Inn.	Lung	Abdomen Inn.	Carcass Inn.	Result
Control group										
LM 2/z	+	+	+	-	+	+	+	+	-	Pos.
LM 4/z	+	-	+	-	-	-	-	-	-	Pos.
LM 5/z	+	-	-	-	-	-	-	-	-	Pos.
LM 7/z	+	-	+	-	-	+	-	-	-	Pos.
LM 8/z	-	-	+	-	-	-	-	-	+	Pos.
LM 9/z	+	-	+	-	-	-	-	-	-	Pos.
LM 11/z	-	-	-	-	-	-	-	-	-	Neg.
LM 12/z	+	-	-	-	-	-	-	-	-	Pos.
LM 15/z	-	-	-	-	-	-	-	-	-	Neg.
LM 20/z	-	-	+	+	-	+	-	-	-	Pos.
LM 24/z	+	-	+	-	-	-	-	-	-	Pos.
LM 28/z	-	-	+	-	-	-	-	-	-	Pos.
LM 31/z	-	-	+	-	-	+	+	-	-	Pos.
LM 33/z	-	-	+	-	-	+	+	-	-	Pos.
	7/14	1/14	10/14	1/14	1/14	5/14	3/14	1/14	1/14	
Vaccine group										
LM 1/z	+	-	+	-	-	+	-	-	-	Pos.
LM 3/z	-	-	-	-	-	-	-	-	-	Neg.
LM 10/z	-	-	+	-	-	+	-	-	-	Pos.
LM 14/z	+	-	+	+	-	-	-	-	-	Pos.
LM 16/z	+	-	-	-	-	-	-	-	-	Pos.
LM 18/z	-	-	-	-	-	-	-	-	-	Neg.
LM 19/z	-	-	+	-	-	+	+	-	-	Pos.
LM 21/z	+	-	+	-	-	-	-	-	-	Pos.
LM 23/z	-	-	-	-	-	-	-	-	-	Neg.
LM 25/z	-	-	+	-	-	-	-	-	-	Pos.
LM 27/z	+	-	-	-	-	-	-	-	-	Pos.
LM 29/z	-	-	+	-	-	-	-	-	-	Pos.
LM 30/z	+	-	-	-	-	-	-	-	-	Pos.
LM 32/z	+	+	+	+	+	+	+	+	+	Pos.
LM 35/z	-	-	-	-	-	+	-	-	-	Pos.
	7/15	1/15	8/15	2/15	1/15	5/15	2/15	1/15	1/15	

Table 8: The time interval between the start of incubation in an incubator and the positive identification of the first *Mycobacterium bovis* colonies from the different specimens.

Number	**Tonsils**	**Head Inn.**	**Thoracic Inn.**	**Abdominal Inn.**	**Lung**	**Carcass Inn.**	**Final result**
Control							
LM 2/z	3 wks	4 wks	4 wks	5 wks	4 wks	no growth	Positive
LM 4/z	3 wks	3 wks	no growth	no growth	no growth	no growth	Positive
LM 5/z	5 wks	no growth	no growth	no growth	no growth	no growth	Positive
LM 7/z	2 wks	2 wks	3 wks	no growth	no growth	no growth	Positive
LM 8/z	no growth	2 wks	no growth	no growth	no growth	2 wks	Positive
LM 9/z	5 wks	3 wks	no growth	no growth	no growth	no growth	Positive
LM 11/z	no growth	no growth	no growth	no growth	no growth	no growth	Negative
LM 12/z	6 wks	no growth	no growth	no growth	no growth	no growth	Positive
LM 15/z	no growth	no growth	no growth	no growth	no growth	no growth	Negative
LM 20/z	no growth	2 wks	9 wks	no growth	no growth	no growth	Positive
LM 24/z	5 wks	5 wks	no growth	no growth	no growth	no growth	Positive
LM 28/z	no growth	4 wks	no growth	no growth	no growth	no growth	Positive
LM 31/z	no growth	3 wks	6 wks	no growth	4 wks	no growth	Positive
LM 33/z	no growth	2 wks	3 wks	no growth	3 wks	no growth	Positive
Vaccine							
LM 1/z	3 wks	3 wks	4 wks	no growth	no growth	no growth	Positive
LM 3/z	no growth	no growth	no growth	no growth	no growth	no growth	Negative
LM 10/z	no growth	4 wks	4 wks	no growth	no growth	no growth	Positive
LM 14/z	3 wks	3 wks	3 wks	no growth	no growth	no growth	Positive
LM 16/z	no growth	12 wks	no growth	no growth	no growth	no growth	Positive
LM 18/z	no growth	no growth	no growth	no growth	no growth	no growth	Negative
LM 19/z	no growth	3 wks	3 wks	no growth	3 wks	no growth	Positive
LM 21/z	2 wks	2 wks	no growth	no growth	no growth	no growth	Positive
LM 23/z	no growth	no growth	no growth	no growth	no growth	no growth	Negative
LM 25/z	no growth	3 wks	no growth	no growth	no growth	no growth	Positive
LM 27/z	8 wks	no growth	no growth	no growth	no growth	no growth	Positive
LM 29/z	no growth	5 wks	no growth	no growth	no growth	no growth	Positive
LM 30/z	8wks	no growth	no growth	no growth	no growth	no growth	Positive
LM 32/z	2 wks	2 wks	2 wks	2 wks	2 wks	2 wks	Positive
LM 35/z	no growth	no growth	10 wks	no growth	no growth	no growth	Positive

ANNEXURE III

1. BODY MASS OF DISEASED VERSUS HEALTHY ANIMALS

Methods for analyses

After some literature studying Crawley (1996) states the following "If the response variable takes the form of a percentage change in some continuous measure then this data are probably better treated by analysis of covariance using final weight as the response variable and initial weight as the covariate, or by specifying the response variable to be the logarithm of the ratio of final weight to initial weight.

The latter one is the one that I tested using an ANOVA and this ratio then corrects for the differences in initial weight and the log corrects for any non-normality. Please find results below.

Results

There were no significant differences found in the response variable for neither the sick versus healthy animals (variable X0) or between the different treatments (Control vs LD vs HD) for either the first (bm1) or the second study (bm2).

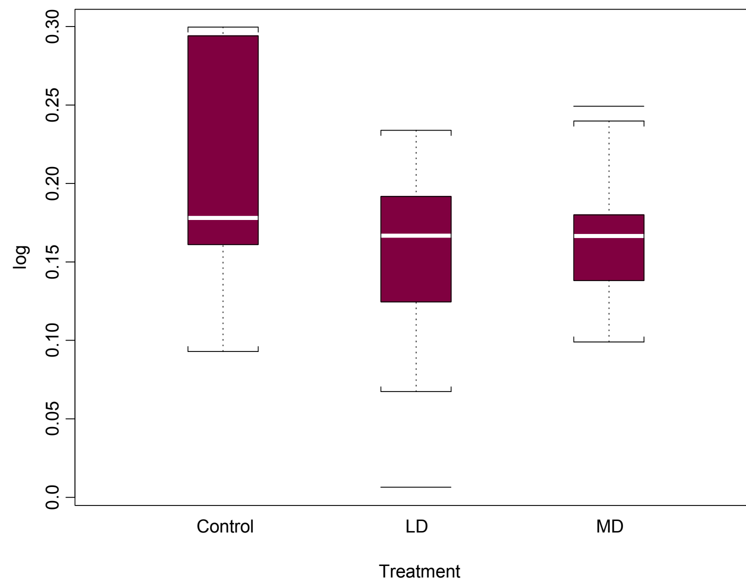
1.1 Study 1: Infection Model

Terms:

	X0	Treatment	Residuals
Sum of Squares	0.00292144	0.00855476	0.09360286
Deg. of Freedom	1	2	22

Residual standard error: 0.06522787
Estimated effects may be unbalanced.

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
X0	1	0.00292144	0.002921444	0.686643	0.4162083
Treatment	2	0.00855476	0.004277379	1.005336	0.3821219
Residuals	22	0.09360286	0.004254675		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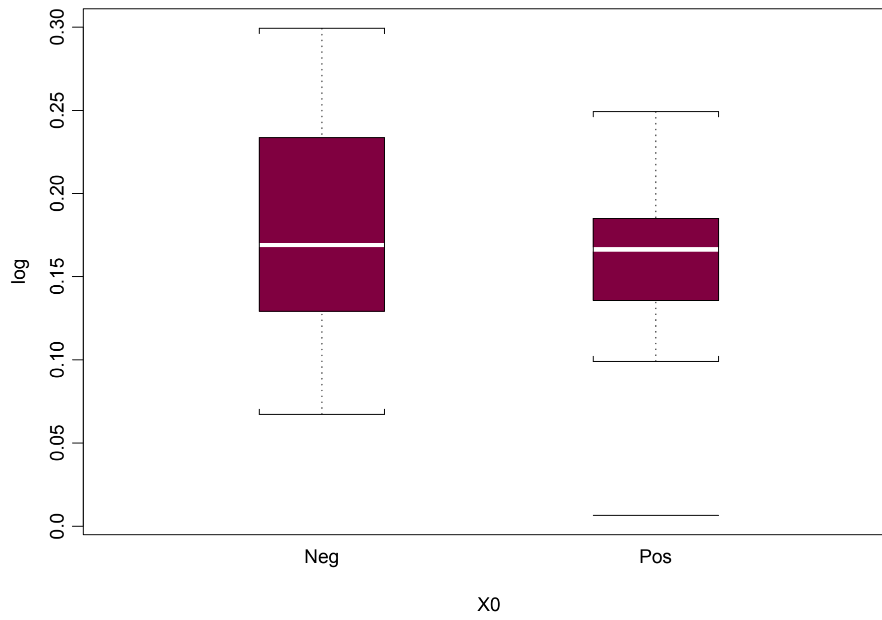
0.0
0.05
0.10
0.15
0.20
0.25
0.30
Control
LD
MD
Treatment


log
0.0
0.05
0.10
0.15
0.20
0.25
0.30
Neg
Pos
X0

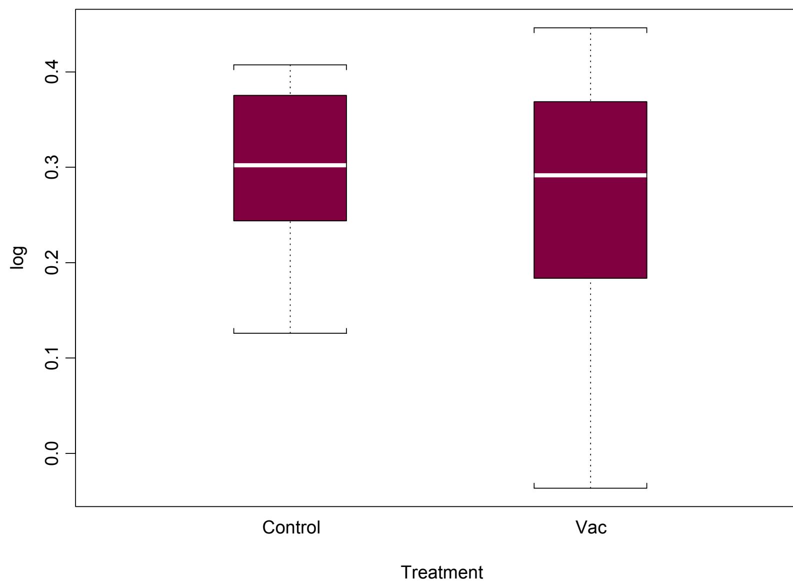
1.2 Study 2: BCG vaccine study

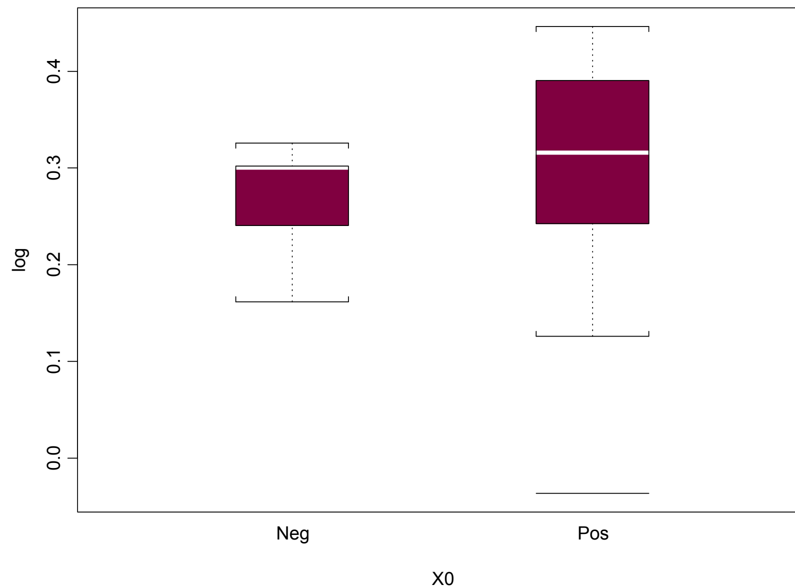
Terms:

	X0	Treatment	Residuals
Sum of Squares	0.0022674	0.0054788	0.2747677
Deg. of Freedom	1	1	20

Residual standard error: 0.1172109
Estimated effects may be unbalanced.

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
X0	1	0.0022674	0.00226743	0.1650433	0.6888692
Treatment	1	0.0054788	0.00547879	0.3987945	0.5348644
Residuals	20	0.2747677	0.01373838		





2. BLOOD PARAMETERS IN HEALTHY VERSUS SICK ANIMALS

Methods

As the response variables are all continuous variables and we were interested to see whether there was any significant differences between the positive and negative individuals over the specified time period an ANOVA could be used. The different samples taken at different times per individuals were averaged to obtain one mean value for each animal for each parameter. The different values over time could not be used as replicates because of the problem of pseudoreplication. Each parameter was first checked for normality and when there was deviation from this the parameter was transformed either by using a log transformation or by using the arcsine square root transformation.

Results

For both the first (haem1) and the second (haem2) study there were no significant differences in any of the parameters between the positive and negative individuals.

Please see the ANOVA results below. The box plots were not done but this can be done if wanted. Where a variable has a log at the start of the name it means this variable was log transformed and where the arcsine is in front it means that the arcsine square root transformation was used. The status variable is the factor of positive or negative status of the animal.

2.1 Haematology study 1

2.1.1 Parameter **WBC**

Terms:

	Status	Residuals
Sum of Squares	0.01192	81.87487
Deg. of Freedom	1	26

Residual standard error: 1.774552
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.01192	0.011919	0.003785099	0.9514131
Residuals	26	81.87487	3.149033		

2.1.2 Parameter **RBC**

Terms:

	Status	Residuals
Sum of Squares	1.9839	43.9663
Deg. of Freedom	1	26

Residual standard error: 1.300389
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	1.9839	1.983897	1.173201	0.2886875
Residuals	26	43.9663	1.691012		

2.1.3 Parameter **Hgb.g/dl**

Terms:

	Status	Residuals
Sum of Squares	3.83478	64.45487
Deg. of Freedom	1	26

Residual standard error: 1.574495
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	3.83478	3.834777	1.546884	0.2246935
Residuals	26	64.45487	2.479033		

2.1.4 Parameter **Hct**

Terms:

	Status	Residuals
Sum of Squares	21.1655	494.9642
Deg. of Freedom	1	26

Residual standard error: 4.363151
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	21.1655	21.16547	1.111802	0.301391
Residuals	26	494.9642	19.03708		

2.1.5 Parameter **MCHC g/dl**

Terms:

	Status	Residuals
Sum of Squares	1.57808	14.02299
Deg. of Freedom	1	26

Residual standard error: 0.7344018
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	1.57808	1.578077	2.925908	0.09907662
Residuals	26	14.02299	0.539346		

2.1.6 ‘Arcsinsqrt **MCV**

Terms:

	Status	Residuals
Sum of Squares	0.001091	1.588183
Deg. of Freedom	1	26

Residual standard error: 0.2471517
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.001091	0.00109149	0.01786869	0.8946898
Residuals	26	1.588183	0.06108394		

2.1.7 ‘Arcsinsqrt **PLT**

Terms:

	Status	Residuals
Sum of Squares	0.0080326	0.5061771
Deg. of Freedom	1	26

Residual standard error: 0.139529

Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.0080326	0.00803264	0.4126001	0.5262766
Residuals	26	0.5061771	0.01946835		

2.1.8 Parameter **Lymph**

Terms:

	Status	Residuals
Sum of Squares	5.4418	397.6667
Deg. of Freedom	1	26

Residual standard error: 3.910866
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	5.4418	5.44183	0.3557946	0.5560096
Residuals	26	397.6667	15.29487		

2.1.9 Parameter **MCH**

Terms:

	Status	Residuals
Sum of Squares	0.04297	34.16417
Deg. of Freedom	1	26

Residual standard error: 1.146301
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.04297	0.042972	0.03270283	0.857896
Residuals	26	34.16417	1.314007		

2.2 Haematology study 2

2.2.1 **LogWBC**

Terms:

	Status	Residuals
Sum of Squares	0.051204	1.318171
Deg. of Freedom	1	27

Residual standard error: 0.2209551
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.051204	0.05120422	1.048812	0.3148694
Residuals	27	1.318171	0.04882116		

2.2.2 Parameter **RBC**

Terms:

	Status	Residuals
Sum of Squares	0.65794	26.56006
Deg. of Freedom	1	27

Residual standard error: 0.9918195
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.65794	0.6579375	0.6688355	0.4206169
Residuals	27	26.56006	0.9837060		

2.2.3 Parameter **Hgb. g.dl**

Terms:

	Status	Residuals
Sum of Squares	1.57059	55.78072
Deg. of Freedom	1	27

Residual standard error: 1.437342
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	1.57059	1.570588	0.7602245	0.3909459
Residuals	27	55.78072	2.065952		

2.2.4 Parameter **Hct**

Terms:

	Status	Residuals
Sum of Squares	13.1290	564.7799
Deg. of Freedom	1	27

Residual standard error: 4.573595
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	13.1290	13.12904	0.62765	0.4351246
Residuals	27	564.7799	20.91777		

2.2.5 Parameter **MCV**

Terms:

	Status	Residuals
Sum of Squares	0.0132	117.1634
Deg. of Freedom	1	27

Residual standard error: 2.083119
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.0132	0.013170	0.003035078	0.9564712
Residuals	27	117.1634	4.339386		

2.2.6 Parameter **MCH.pg**

Terms:

	Status	Residuals
Sum of Squares	0.00556	10.74593
Deg. of Freedom	1	27

Residual standard error: 0.6308705
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.00556	0.0055632	0.01397802	0.9067617
Residuals	27	10.74593	0.3979975		

2.2.7 Parameter **MCHC**

Terms:

	Status	Residuals
Sum of Squares	0.6299	149.4167
Deg. of Freedom	1	27

Residual standard error: 2.352435
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.6299	0.629917	0.1138278	0.7384365
Residuals	27	149.4167	5.533952		

2.2.8 Parameter **PLT**

Terms:

	Status	Residuals
Sum of Squares	11380	21725994
Deg. of Freedom	1	27

Residual standard error: 897.032
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	11380	11379.8	0.01414231	0.906218
Residuals	27	21725994	804666.5		

2.2.9 **LogLymph**

Terms:

	Status	Residuals
Sum of Squares	0.052190	1.116981
Deg. of Freedom	1	27

Residual standard error: 0.2033954
Estimated effects may be unbalanced

	Df	Sum of Sq	Mean Sq	F Value	Pr (F)
Status	1	0.052190	0.05219016	1.261556	0.2712451
Residuals	27	1.116981	0.04136967		

3. STATISTICAL INTERPRETATION OF THE MACROSCOPIC, HISTOPATHOLOGY AND MYCOBACTERIAL CULTURE RESULTS OF STUDY 2

As this data was binomial data meaning that the response variables were either positives or negatives a logistic regression was performed on the data. The response variables looked at were culture positive, macroscopic positive and histopathology positive. The factor whether the subject was vaccinated or not was added in as an independent variable. The aim was to look whether there was a significant effect of the vaccine on the response variables.

The way that one determines whether this was significant or not is to run the model and then take out this factor. The change in deviance associated with removal of this factor and the number of degrees of freedom are then compared against chi squared critical values. In order for one to have a 95% significance with 1 degree of freedom the change in deviance has to exceed 3.841. In none of these analysis was the deviance greater than this amount so there is not significant difference between the vaccinated or control groups for the response variables tested.

3.1 Response variate: Culture positive

Binomial totals: Subject1
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, Vac_cont

*** Summary of analysis ***

	d.f.	deviance	mean deviance	deviance ratio
Regression	1	0.10	0.102	0.10
Residual	26	26.17	1.007	
Total	27	26.28	0.973	
Change	-1	-0.10	0.102	0.10

* MESSAGE: ratios are based on dispersion parameter with value 1

**** Regression Analysis *****

3.2 Response variate: Macro positive

Binomial totals: Subject1
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, Vac_cont

*** Summary of analysis ***

	d.f.	deviance	mean deviance	deviance ratio
Regression	1	1.47	1.467	1.47
Residual	26	36.78	1.414	
Total	27	38.24	1.416	
Change	-1	-1.47	1.467	1.47

* MESSAGE: ratios are based on dispersion parameter with value 1

***** Regression Analysis *****

3.3 Response variate: Histopathology positive

Binomial totals: Subject1
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, Vac_cont

*** Summary of analysis ***

	d.f.	deviance	mean deviance	deviance ratio
Regression	1	0.74	0.745	0.74
Residual	26	36.78	1.414	
Total	27	37.52	1.390	

Change	-1	-0.74	0.745	0.74

* MESSAGE: ratios are based on dispersion parameter with value 1

For the number of active lesions the model fitted was a log linear regression as the response variable was a count. The test for significance is the same as that explained above. There was still 1 degree of freedom and a change in deviance of 2.20 was obtained, which is smaller than 3.841 so one can deduce that the vaccine does not significantly affect the number of active lesions.

**** Regression Analysis *****

3.4 Response variate: Active lesion
Distribution: Poisson
Link function: Log
Fitted terms: Constant, Vac_cont

*** Summary of analysis ***

	d.f.	deviance	mean deviance	deviance ratio
Regression	1	2.20	2.200	2.20
Residual	26	64.22	2.470	
Total	27	66.42	2.460	
Change	-1	-2.20	2.200	2.20

* MESSAGE: ratios are based on dispersion parameter with value 1