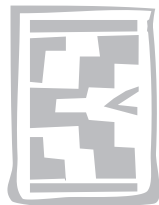

An experimental intratonsilar infection model for bovine tuberculosis in African buffaloes, *Syncerus caffer*

L. DE KLERK[1], A.L. MICHEL[2], D.G. GROBLER[1], R.G. BENGIS[3], M. BUSH[4], N.P.J. KRIEK[5], M. S. HOFMEYR[1], J.F.T. GRIFFIN[6] and C.G. MACKINTOSH[7]

ABSTRACT

DE KLERK, L., MICHEL, A.L., GROBLER, D.G., BENGIS, R., BUSH, M., KRIEK, N.P.J., HOFMEYR, M.S., GRIFFIN, J.F.T. & MACKINTOSH, C.G. 2006. An experimental intratonsilar infection model for bovine tuberculosis in African buffaloes, *Syncerus caffer*. *Onderstepoort Journal of Veterinary Research*, 73:293–303

An infection model for *Mycobacterium bovis* in African buffaloes, *Syncerus caffer*, was developed, using the 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 cfu (high dose) of *M. bovis* strain isolated from a buffalo. A control group of six buffaloes received saline via the same route. The infection status was monitored *in vivo* using the comparative intradermal tuberculin test, and *in vitro* by the modified interferon-gamma assay. All buffaloes were euthanazed 22 weeks post infection and lesion development was assessed by macroscopic examination, culture and histopathology. It was found that the high dose caused macroscopic lesions in nine out of 11 buffaloes. *Mycobacterium bovis* was isolated from all buffaloes in the high-dose group and from six out of 11 in the low-dose group.

Keywords: African buffalo, Bacille Calmette-Guérin, bovine tuberculosis, intratonsilar infection model, Kruger National Park, *Mycobacterium bovis, Syncerus caffer*

INTRODUCTION

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, was first diagnosed in free-ranging buffaloes, *Syncerus caffer*, in the Kruger National Park in 1991 (KNP) (Bengis, Kriek, Keet, Raath, De Vos & Huchzermeyer 1996). In this ecosystem, buffaloes are now considered to be the main reservoir and maintenance host (De Vos, Bengis, Kriek, Michel, Keet, Raath & Huchzermeyer 2001). Since the initial diagnosis, BTB infection has spilled over into a variety of other species (Keet, Kriek, Penrith, Michel & Huchzermeyer 1996) and has been confirmed in kudus, *Tragelaphus strepsiceros*, baboons, *Papio ursinus*, lions *Panthera leo*, leopards, *Panthera pardus*, hyenas, *Crocuta crocuta*, cheetahs, *Acinonyx jubatus*, warthogs, *Phacochoerus ethiopicus*, genets, *Genetta genetta* and honey badgers, *Mellivora capensis* (Keet 1996; Keet, Kriek, Bengis, Grobler & Michel 2000; Keet, Kriek, Bengis & Michel 2001; Michel 2002).

Because of the number of species infected with BTB, the control and eventual eradication of BTB

[1] South African National Parks, Private Bag X402, Skukuza, 1350 South Africa

[2] ARC-OVI, Private Bag X05, Onderstepoort, 0110 South Africa

[3] Office of the State Veterinarian, Kruger National Park, P.O. Box 12, Skukuza, 1350 South Africa

[4] Chief of Veterinary Services, Conservation and Research Center, Smithsonian Institution, Front Royal, Virginia, USA

[5] Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[6] Disease Research Laboratory, Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand

[7] Deer Research Unit, AgResearch, Invermay Agriculture Research Centre, Mosgiel, New Zealand

Accepted for publication 3 September 2006—Editor

from the KNP will become increasingly difficult. Complicating factors include the widespread geographical ranges occupied by infected buffalo herds and the potential of other species to become maintenance hosts. The spread of infection both within and between herds is thought to be further enhanced by the gregarious lifestyle of buffaloes, as well as the dynamic fragmentation and coalescing of buffalo herds (De Vos *et al.* 2001). Various strategies to control and eventually eradicate the disease from the KNP have been considered. Of the strategies that may be effective, vaccination could be the most feasible option provided a vaccine candidate can be validated in an experimental buffalo model.

The Bacille Calmette-Guérin (BCG) vaccine is the only vaccine currently available with proven safety and efficacy for the control of the infection in humans. The BCG vaccine has also been tested in a number of domesticated and wild species (Buddle, Keen, Thomson, Jowett, McCarthy, Heslop & De Lisle 1995; Buddle, Skinner & Chambers 2000; Corner, Buddle, Pfeiffer & Morris 2001, 2002). Variability in the efficacy seen in these experimental studies has precluded the use of the BCG as a vaccine for domestic livestock or wildlife. Factors that affected the efficacy of BCG vaccine in experimental protocols included the age of the animals that were vaccinated, prior sensitization to environmental mycobacteria (Buddle, Wards, Aldwell, Collins & De Lisle 2002), delivery and dosage, and whether single or multiple booster doses were used (Griffin, Mackintosh, Slobbe, Thomson & Buchan 1999; Cross, Labes, Griffin & Mackintosh 2000). When a pathology scoring system was recently applied to cattle which had been experimentally challenged with *M. bovis*, BCG vaccination reduced disease severity by 75 % (Vordermeier, Chambers, Cockle, Whelan, Simmons & Hewinson 2002). The vaccine also gave excellent protection to red deer, *Cervus elaphus*, at the Disease Research Laboratory (DRL) in Dunedin, New Zealand, if a booster was administered 8 weeks after the initial BCG vaccination (Griffin, Chinn, Rodgers & Mackintosh 2001). An added advantage of vaccination, even if the BCG vaccine does not provide full protection against the disease, is that it appears to reduce the severity of the disease and subsequent mycobacterial excretion. Reduced contamination of the environment could also limit the subsequent spread of infection (Cross *et al.* 2000). Indeed, under certain environmental conditions, *M. bovis* can survive outside its host for long enough to significantly increase the likelihood that other animals may become infected and develop disease (Tanner & Michel 1999).

To evaluate the efficacy of such a vaccine in buffaloes, a reliable infection model had to be developed which mimics natural infection in this species. The model should reproduce the typical range of lesions, progression of the disease and the immunological response seen in naturally infected animals (Mackintosh, Waldrup, Labes, Buchan & Griffin 1995). The model should be also repeatable, practical to execute, safe and economical.

Various methods of establishing experimental infection with *M. bovis* have been studied in different host species. The intra-tracheal route of infection has been used in cattle (Buddle *et al.* 1995), possums (Corner *et al.* 2002), the oral route in ferrets (Cross *et al.* 2000) and the intratonsilar inoculation in red deer (Mackintosh *et al.* 1995; Griffin, Mackintosh & Buchan 1995) and cattle (Palmer, Whipple, Rhyan, Bolin & Saari 1999). The intratonsilar method was selected for this study because it is easy to execute, safe and economical. It involves instilling 0.2 mℓ of an inoculum into the left tonsilar crypt while the experimental animal is under anaesthesia. The disease in infected cattle and deer mimicked the natural disease in terms of its pathogenesis and rate of progression and severity of lesions in deer (Griffin *et al.* 1995) and cattle (Palmer *et al.* 1999). The aim of this study is to establish an *M. bovis* experimental model of infection in buffaloes in order to evaluate further BTB vaccine candidates.

MATERIAL AND METHODS

Animals

Twenty-eight buffalo calves, varying in age from 12–24 months, were captured in a tuberculosis-free area of the KNP. The calves were randomly divided into three groups, with comparable numbers of bull and heifer calves in each group. The two experimental groups comprised eleven animals each while the control group comprised six animals. The three groups were housed separately. The control group was placed in a facility furthest away from the group that received the higher infectious dose of virulent *M. bovis*.

Husbandry and monitoring

The calves were housed in bomas of 600 m^2 with an inner and an outer fence designed to keep out predators. The animals were observed three times a day, and interactions, general health, and condition scores were recorded. Injuries and acute illnesses were treated appropriately. They were fed twice a

day with teff hay and lucerne mixed at a ratio of 2:1 and water was available *ad libidum.* At capture all the calves were tested with the comparative intradermal tuberculin skin test as well as the gamma-interferon assay. All calves tested negative for bovine tuberculosis although sensitization to environmental mycobacteria was seen in some animals. After 4 weeks the gamma-interferon assays were repeated and all the results were comparable to those obtained at capture.

Mycobacterium bovis strain

During a survey in 1998 to determine the prevalence of BTB in buffaloes in the KNP, suspect tissue samples were submitted for culture (Rodwell, Kriek, Bengis, Whyte, Viljoen, De Vos & Boyce 2001). Mycobacterial isolates were identified using biochemical and PCR tests followed by restriction fragment length polymorphism (RFLP) characterization 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 the trial. Subcultures of this 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 microscopically counting of serial dilutions in a Neubauer counting chamber to 3 x 10^2 (low-dose inoculum) and 3 x 10^4 (high-dose inoculum) per 200 µℓ, respectively. Tween 80 was used to avoid clumping of bacteria, allowing for accurate counting. Aliquots of each serial dilution were plated out in triplicate onto Petri dishes containing Lowenstein-Jensen medium with pyruvate. To avoid desiccation during the prolonged incubation at 37 °C, Petri dishes were sealed and placed in a humid chamber for 10 weeks.

Anaesthesia

A combination of etorphine hydrochloride (M99, Novartis SA Animal Health) and xylazine (Chanazine 2 %, Centaur, Bayer Animal Health) at standard dosages used for routine buffalo immobilization was used to anaesthetize the buffalo calves. Xylazine was used because it is a good muscle relaxant and it enhanced the relaxation of the jaw muscles, facilitating the opening of the mouth during the infection procedure. During the rest of the study period, a combination of M99 and azaperone (Stresnil, Janssen Animal Health) was used as described previously (Bengis & Raath 1993).

Experimental infection procedure

The control animals were handled first. Blood samples were collected from the jugular vein into vacuum tubes containing appropriate solutions for preservation and/or preventing coagulation, as required. The calves were then rolled onto strong tarpaulin stretchers and moved to a separate pen to reduce the likelihood of contact with the animals infected with live *M. bovis*.

The two groups to be infected, each comprising 11 calves, were anaesthetized and inoculated with live *M. bovis* culture material as follows. The anaesthetized calves were placed in sternal recumbency with their heads lowered to allow any oral fluid resulting from the administration of xylazine, to drain before the instillation of the bacterial suspension into the left tonsilar crypt. Each animal's mouth was opened and its tongue was reflected to the left side of the operator. The base of the tongue was depressed using a 400 mm laryngoscope, so that the left tonsilar crypt could be seen. The *M. bovis* suspension was instilled into the crypt with a 1 mℓ syringe fitted with a 300 mm long 18 G needle with a ball tip. Any spillage or haemorrhage from the crypt following instillation was recorded. The eleven calves in the low-dose group received 0.2 mℓ of a suspension containing about 3 x 10^2 cfu of *M. bovis* and the 11 calves in the high-dose group received 0.2 mℓ of a suspension containing 3 x 10^4 cfu of *M. bovis*. The six calves in the control group received 0.2 mℓ of saline into the left tonsilar crypt following the same overall procedure.

After the procedure was completed, the animals were revived by administering the antidote diprenorphine hydrochloride (M50/50, Novartis SA Animal Health) at twice the dosage of the M99. Whenever xylazine was used to immobilize the calves, 3–5 mℓ of yohimbine were also administered. The different experimental groups of calves were kept in separate bomas.

Laboratory tests

Interferon-γ assay (Bovigam)

The interferon-γ assay (IFN-γ) is a rapid, blood-based assay of cell-mediated immunity (CMI) used for the diagnosis of BTB in cattle (Woods *et al.* 1991). However, when used for the diagnosis in buffaloes there was a lack of specificity (Michel *et al.* 2000).

Through subsequent modification of the commercial kit protocol into a triple comparative test (i.e. use of *Mycobacterium fortuitum* besides *Mycobacterium avium* and *M. bovis* purified protein derivatives [PPD], as an ancillary antigen), discrimination between specific and non-specific immune reactions was significantly improved (Grobler *et al.* 2002). In this experiment the modified protocol was used as described previously by Grobler *et al.* (2002).

Whole blood count

Whole blood was collected in an EDTA tube from every animal and assayed within 6 h of blood collection to perform whole blood counts on the T-890 coulter counter (Beckman Coulter). Blood smears were prepared on glass slides, stained with Diff-Quick (Kyro-quick, Kyron Laboratories) and examined for *Babesia* and *Theileria* parasites.

Bacteriology

Specimens for culture were collected from the following lymph nodes irrespective of whether lesions were present. The left and right tonsils and both medial retropharyngeal lymph nodes were processed separately and equally divided for mycobacterial culture and histopathology. All lesions detected in any other of the lymph nodes or organs, were collected and processed for mycobacterial culture and histopathology. Specimens from other lymph nodes (as indicated below) were pooled for culture:

- Pooled head lymph nodes — Mandibular and parotid lymph nodes
- Pooled thoracic lymph nodes — Mediastinal and bronchial lymph nodes
- Pooled abdominal lymph nodes — Mesenteric, hepatic, renal, omasal and abomasal lymph nodes
- Pooled carcass lymph nodes — Cervical, prescapular, axillary, popliteal, inguinal and mammary or scrotal lymph nodes.

Samples were transferred to and processed at the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute, where they were cultured and identified as described by Bengis *et al.* (1996). Cultures were considered negative for *M. bovis* when no growth was detected after 12 weeks of incubation. In this study the culture result was used as the gold standard to indicate if an animal was infected with *M. bovis.* The same isolation and identification protocols were applied to swabs that were taken during the course of infection.

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 4–6 µm were routinely stained with haematoxylin and eosin, and selected sections with the Ziehl-Neelsen acid-fast stain. The Ziehl-Neelsen stained histopathology sections were examined microscopically for the presence of acid-fast bacilli (AFB).

Intradermal tuberculin test

A comparative intradermal tuberculin test using 0.1 mℓ of bovine PPD (0.1 mg/mℓ) and 0.1 mℓ of avian PPD (0.05 mg/mℓ, Lelystad, The Netherlands) was done on all the calves 6 weeks prior to infection and again 3 months after infection. Injection sites on both sides of the lower neck were shaved with a battery-operated hair clipper. As a rule, bovine tuberculin PPD was injected intradermally on the left and avian PPD on the right side of the neck with a McClintok syringe. The skin thickness was measured with a calliper before and 72 h after injection. Both manipulations necessitated the calves to be anaesthetized, firstly to give the injections and secondly, to palpate and assess the nature of the skin reaction.

The intradermal tuberculin test results were interpreted as recommended by the OIE:

- Increase of skin thickness in response to injection of bovine tuberculin PPD < 2 mm = negative

Provided the reaction to bovine tuberculin PPD was greater than that elicited by the avian tuberculin PPD the following interpretation was applied:

- If the increase in skin thickness at the bovine tuberculin injection site minus the increase at the avian injection site is 2–4 mm, the result is suspect.
- If the increase in skin thickness at the bovine tuberculin injection site minus the increase at the avian injection site is > 4 mm, the result is positive.

Routine monitoring

After infection, the calves were monitored three times daily for evidence of clinical disease or abnormal behaviour. No acute onset of disease was observed.

Blood specimens, nasal swab collection and mass gain during he course of infection

Apart from the day of experimental *M. bovis* infection and euthanasia, the buffalo calves were anaesthetized at three further occasions between weeks 5 and 15 post infection (p.i.), at which time they were weighed, and blood specimens and nasal swabs were collected. Whole blood was collected from each experimental animal and preserved in heparin as well as in EDTA for the IFN-γ assay and routine haematology. Vacuum tubes without preservative were used to collect serum for serum banking. Nasal swabs were collected for bacteriology. On the day of euthanasia the same sampling procedures were applied as mentioned previously. In total, five sets of samples were collected for each individual, the last being taken on the day of euthanasia.

Each anaesthetized calf was weighed regularly by being lifted onto a tarpaulin stretcher that was connected to a scale attached to a hydraulic crane. The scale was zeroed before each calf was weighed. The mass of each calf was recorded to reflect its change in mass over the duration of the experiment.

Necropsy procedure

All the experimental animals were euthanazed with succinyl dicholine chloride (scoline) and then immediately subjected to a detailed necropsy in the abattoir at Skukuza. Five animals (two each from the low and high-dose groups and one from the control group) were necropsied 18 weeks after infection, to establish whether the infection technique developed for deer was valid in buffaloes. Previous studies showed that characteristic pathological changes are evident in deer 18 weeks after experimental infection (Griffin, unpublished data 2002). At the end of the study, 22 weeks p.i., all the remaining calves were euthanazed.

Selected lymph nodes were removed from the carcass and after being thinly sliced with a scalpel blade, were visually examined for the presence of macroscopic tuberculous lesions. The tonsils and the mandibular, parotid, and medial retropharyngeal lymph nodes together with the mediastinal and bronchial lymph nodes and the pre-scapular, axillary, popliteal, inguinal, mammary or scrotal, hepatic, ruminal, omasal, abomasal, mesenteric, ileo-caecal and rectal lymph nodes were collected. The medial retropharyngeal lymph nodes of each animal were labelled and photographed.

The lungs were initially carefully palpated to detect any nodules or lesions, after which they were systematically sliced with a knife to detect any lesions. All lesions that were detected in the lungs, lymph nodes and tonsils were collected and submitted for histopathology and bacterial culture. Irrespective of whether macroscopic lesions were detected in specimens of the tonsils and lymph nodes listed above, these tissues were collected for histopathology and culture. Specimens for histopathology were preserved in 10 % buffered formalin and submitted to the Department of Pathology, of the Faculty of Veterinary Science, University of Pretoria at Onderstepoort, for processing and examination.

Grading of macroscopic lesions

The following criteria were used to grade the macroscopic lesions of each individual animal:

Grade 0 – No visible lesions

Grade 1 – Less than 50 % of a single lymph node or tonsil affected

Grade 2 – More than 50 % of a single lymph node or tonsil affected

Grade 3 – Two or three lymph nodes and/or tonsils involved

Grade 4 – More than three lymph nodes involved

Grade 5 – Multiple lymph nodes as well as organs involved (Miliary disease)

Statistical methods

An ANOVA was used because the response variables were all continuous variables and the aim was to determine whether there were any significant differences between the positive and negative individuals over the specified time period. This method of analysis was used for both the haematology parameters and the body mass data sets.

RESULTS

***Mycobacterium bovis* inoculum**

Plate counts of serial dilutions of the *M. bovis* inoculum 6 weeks after experimental infection revealed that the infectious doses administered were 3.2 x 10^2 and 3 x 10^4 cfu, respectively.

Interferon-γ assay

The results are shown in Table 1. All blood samples collected on the day of the experimental infection (Day 0) tested negative for bovine PPD. Five weeks p.i. seven out of 11 animals inoculated with the high dose tested positive with IFN-γ. All control calves as

TABLE 1 Results of the gamma-interferon assay from the control, low dose and high dose group animals

No.	Day 0	Week 5	Week 11*	Week 15	Week 21/22
Control					
LM4	–	–	–	–	AV
LM5	–	–	Invalid	–	–
LM8	–	–	–	+	–
LM9	–	–	–	AV	AV
LM18	–	–	–	–	–
LM26	–	–	–	–	–
Low dose					
LM1	–	–	–	+	+
LM2	–	–	–	–	–
LM7	–	–	–	Invalid	–
LM12	–	–	+	–	–
LM14	–	–	–	–	–
LM17	–	–	+	+	+
LM21	–	–	–	–	+
LM22	–	–	+	+	+
LM23	–	–	–	–	–
LM24	–	–	–	–	–
LM27	–	–	–	+	–
High dose					
LM3	–	–	+	+	+
LM6	–	+	+	+	+
LM10	–	–	+	+	+
LM11	–	+	+	+	+
LM13	–	+	+	+	+
LM15**	–	+	+		
LM16	–	–	–	+	+
LM19**	–	+			
LM20	–	–	+	+	+
LM25	–	+	–	+	+
LM28	–	+	Suspect	+	+

+ (positive) : Bovine – avian (OD) > 0.2
– (negative) : Bovine – avian (OD) < 0.17 provided that bovine (OD) < 0.3
Suspect : Bovine – avian (OD) > 0.17 but < 0.2 provided that bovine (OD) > 0.3
AV (avian reactor) : Avian – bovine (OD) > bovine (OD) + bovine/10
Invalid : Control (OD) > 0.25
Week 11* : Animals were skin tested during Week 11
LM15** & LM19** : Two animals died due to causes unrelated to bovine tuberculosis

well as those receiving the low dose tested negative. The intradermal tuberculin test was applied at 11 weeks p.i. During weeks 13–17 p.i., the number of infected calves testing IFN-γ positive increased until at 22 weeks p.i. all calves in the high-dose group and four out of the 11 in the low-dose group tested positive on IFN-γ. Some non-specific sensitization was observed in all the groups during the trial, but was disregarded, and a false positive result occurred in one animal from the control group after the intradermal tuberculin test.

Whole blood count

Throughout the study whole blood and total lymphocyte counts remained stable for all animals. At 95 % confidence levels no difference could be detected between the haematological parameters of diseased and non-diseased animals

Tuberculin skin test

Two calves tested positive for avian PPD prior to infection (Table 2). The second skin test was carried

TABLE 2 A comparison of the skin tests that were done 2 weeks prior to and 11 weeks post intratonsilar infection, of all three groups of animals from the Infection model (results in mm)

Skin test results											
2 weeks prior to infection						**11 weeks post infection**					
No.	**Bov 0 h**	**Bov 72 h**	**Av 0 h**	**Av 72 h**	**Result**	**Bov 0 h**	**Bov 72 h**	**Av 0 h**	**Av 72 h**	**Result**	**Test site appearance**
Control											
LM 4	18	16	17.7	19.7	Neg.	21.7	23	21.7	23.4	Neg.	Normal
LM 5	15.3	15.5	15.1	16.2	Neg.	17.2	19.5	16	19.4	Neg.	Normal
LM 8	14.4	13.5	14.4	14.3	Neg.	14.6	14.5	14	15	Neg.	Normal
LM 9	13.5	13.8	13.6	19.4	Avian	13.4	15.4	11.6	15.5	Neg.	Normal
LM 18	14.3	14.5	13.9	14.6	Neg.	13.5	15.1	13.8	18.3	Avian	Normal
LM 26	11.3	12.1	11.3	12.3	Neg.	10.1	9.6	10.7	12.8	Neg.	Normal
Low-dose group											
LM 1	12.3	12.5	13.3	18.8	Avian	13.3	31.4	13	15.5	Pos.	Oedema
LM 2	12.5	12.9	13.9	14.9	Neg.	15.2	15.4	15.5	15.3	Neg.	Normal
LM 7	16.3	17.1	15.9	18	Neg.	15.5	16.2	15.9	16.6	Neg.	Normal
LM 12	15.5	15.4	15.2	17	Neg.	15	14.9	15.1	16.5	Neg.	Normal
LM 14	12.4	11.8	13.4	14.2	Neg.	12.6	16.6	12.2	13.4	Susp.	Oedema
LM 17	11.9	12.9	11.8	14	Neg.	11	> 33	10.8	13.1	Pos.	Oedema, necrosis
LM 21	14.5	15.6	15.1	15.8	Neg.	12.8	13.5	15	18.1	Neg.	Small avain nodule
LM 22	13	12.8	14	13.3	Neg.	12.1	> 33	13.3	18.5	Pos.	Oedema, necrosis
LM 23	14.1	14.2	14.4	16.5	Neg.	10.6	12.6	14.2	17	Neg.	Small avian nodule
LM 24	13	13	12.9	13.3	Neg.	12.2	17.5	11.7	12.8	Pos.	Oedema
LM 27	12.4	12	12.5	12.6	Neg.	11.4	16.7	10.7	12.7	Susp.	Oedema
High-dose group											
LM 3	18	17.3	17.3	19.8	Neg.	17.2	> 33	16.4	20.5	Pos.	Oedema, necrosis
LM 6	15	15.7	15.9	15.5	Neg.	16.1	> 33	16.7	17.4	Pos.	Oedema, necrosis
LM 10	13.3	14.8	12.5	14.7	Neg.	13.3	> 33	12.8	17.3	Pos.	Oedema, necrosis
LM 11	11.5	11.4	11.7	11.7	Neg.	10	> 33	11	12.5	Pos.	Oedema, necrosis
LM 13	17.4	18.5	17.1	18.3	Neg.	18.1	> 33	17.3	18.4	Pos.	Oedema, necrosis
LM 16	14.5	14.3	14.4	13.9	Neg.	16.1	> 33	14.8	22.2	Pos.	Oedema, necrosis
LM 20	11.4	10.8	11	10.8	Neg.	10.4	30.4	11.8	14.5	Pos.	Oedema
LM 25	14.5	14.3	14	14.2	Neg.	13	19.7	12.6	14.4	Pos.	Oedema
LM 28	12.4	12	12.5	12.6	Neg.	17.7	> 33	11.5	14.8	Pos.	Oedema, necrosis

Positive : Increase in bovine measurement – increase in avian measurement > 4 mm
Suspect : Increase in bovine measurement – increase in avian measurement > 2 mm; < 4 mm
Avian reactor : Increase in avian measurement – increase in bovine measurement > 4 mm
Negative : Increase in bovine measurement – increase in avian measurement < 2 mm

out 11 weeks p.i. which identified all infected calves in the high-dose group and six of the 11 calves in the low-dose group. None of the control calves tested positive. The increase in skin thickness at the test sites in seven of the nine animals from the high-dose group was no longer measurable because of the pronounced swelling that produced an increase of skin thickness in excess of 33 mm. Many of these skin reactions also demonstrated superficial necrosis covered by a sero-fibrinous exudate. Two of the 11 animals in the low-dose group also had reactions that could not be measured.

Body mass and condition

The average body mass gain was between 0.34 and 0.32 kg per animal per day. No statistically significant difference in body mass gain or condition could be detected between infected and control animals.

Macroscopic lesions and histopathology

Control group

No macroscopic or microscopic lesions were detected in the control animals

Low-dose group

Macroscopic lesions typical for *M. bovis* infection were detected in four out of 11 calves infected with the low dose. Histopathology confirmed BTB infection in these animals. Generally, lymph node lesions varied in size and distribution with small granulomas that had central caseation and calcification.

High-dose group

Two animals in this group died prematurely and were necropsied. LM19 died 5 weeks p.i., but no macroscopic tuberculous lesions were noted. On histological sections of the left retropharyngeal lymph node, a few giant cell granulomas could be seen and numerous small acid-fast bacteria were present in the cytoplasm of multinucleate giant cells. *Mycobacterium bovis* was cultured from lymph node samples. LM15 died at 11 weeks p.i., and mild to moderate lymphadenitis with multiple caseo-necrotic foci was present. Histopathology showed a few acid-fast bacilli in the cytoplasm of the giant cells and epithelioid macrophages. Only one calf (LM16) in the high-dose group did not show macroscopic lesions, while all the remaining calves in this group had varying degrees of caseo-necrotic lymphadenitis. Two calves in the high-dose group (LM16 & LM25) were histopathologically negative for BTB.

The grading of the macropathology was higher in the animals from the high-dose group than in animals from the low-dose group. Generally, a larger percentage of lymph node mass was affected in animals in the high-dose group and histopathology revealed larger numbers of acid-fast bacilli organisms (AFBs) in tissue sections. However, there was no difference in the degree of dissemination of lesions between the two infected groups. Indeed, lesions beyond the left retropharyngeal lymph nodes were only seen in mediastinal lymph nodes in one animal of each of the challenge groups.

DISCUSSION

The experimental procedure described here was successful in inducing an infection in which the tuberculous lesions were comparable to those seen in natural infections of buffaloes in low BTB prevalence herds. The model showed that *M. bovis* could be recovered from at least 5 weeks p.i. from the regional lymph node associated with portal of entry. The overall macroscopic pathology of the experimentally infected animals compared well to the necropsy findings of an adult buffalo with BTB (Keet, Kriek, Huchzermeyer & Bengis 1994). Contrary to findings reported from intra-nasal infections of domestic cattle with 10^7 cfu of live *M. bovis*, none of the buffalo calves in our study developed pulmonary disease (Cassidy, Bryson, Pollock, Evans, Forster & Neill 1998). A direct correlation between severity of disease and infectious dose, described by Cassidy *et al.* (1998), was also demonstrated in our experiment, although much lower infectious doses were used. The grading of the gross pathology of affected tissues revealed significant differences between animals in the high and low-dose groups (Table 3).

In agreement with previous similar *M. bovis* infection experiments by Palmer *et al.* (1999), the retropharyngeal lymph node that drains the palatine tonsil, was the most likely node to develop tuberculous lesions. Lesions were found in the left retropharyngeal lymph nodes of 13 of the 22 infected animals, four and nine from the low-dose and high-dose groups, respectively. *Mycobacterium bovis* was cultured from all the macroscopic lesions submitted for culture. In addition, the macroscopically negative retropharyngeal lymph nodes from two animals in the low-dose group were positive for *M. bovis* on culture. This indicates that viable *M. bovis* can be harboured within the lymphoid tissue without the development of macroscopically detectable lesions for at least 22 weeks following infection.

TABLE 3 Macroscopic pathology, histopathology and culture results of the three different animal groups from the Infection model

No.	Gross pathology			Histopathology	Culture
	Left retro.	Other	Grading	Result (AFB**)	
Control					
LM 4	Neg.	Neg.	0	Neg. (0)	Neg.
LM 5	Neg.	Neg.	0	Neg. (0)	Neg.
LM 8	Neg.	Neg.	0	Neg. (0)	Neg.
LM 9	Neg.	Neg.	0	Neg. (0)	Neg.
LM 18	Neg.	Neg.	0	Neg. (0)	Neg.
LM 26	Neg.	Neg.	0	Neg. (0)	Neg.
Low dose					
LM 1	Pos.	Mediast. ln.	3	Pos. (< 5)	Pos.
LM 2	Neg.	Neg.	0	Neg. (0)	Pos.
LM 7	Neg.	Neg.	0	Neg. (0)	Neg.
LM 12	Neg.	Neg.	0	Neg. (0)	Neg.
LM 14	Neg.	Neg.	0	Neg. (0)	Pos.
LM 17	Pos.	Neg.	2	Pos. (< 5)	Pos.
LM 21	Pos.	Neg.	1	Pos. (< 5)	Pos.
LM 22	Pos.	Neg.	2	Pos. (< 5)	Pos.
LM 23	Neg.	Neg.	0	Neg. (0)	Neg.
LM 24	Neg.	Neg.	0	Neg. (0)	Neg.
LM 27	Neg.	Neg.	0	Neg. (0)	Neg.
High dose					
LM 3	Pos.	Neg.	2	Pos. (0)	Pos.
LM 6	Pos.	Neg.	2	Pos. (< 5)	Pos.
LM 10	Pos.	Mediast. ln.	3	Pos. (0)	Pos.
LM 11	Pos.	Neg.	2	Pos. (0)	Pos.
LM 13	Pos.	Neg.	2	Pos. (> 20)	Pos.
LM 15	Pos.	Neg.	2	Pos. (0)	Pos.
LM 16	Neg.	Neg.	0	Neg. (0)	Pos.
LM 19	Neg.	Neg.	0	Pos. (0)	Pos.
LM 20	Pos.	Neg.	1	Pos. (> 20)	Pos.
LM 25	Pos.	Neg.	1	Neg. (0)	Pos.
LM 28	Pos.	Neg.	2	Pos. (10–20)	Pos.

** The numbers in the histopathology result column indicates the number of acid fast organisms present per slide

At an early stage the IFN-γ test was able to differentiate between infected and non-infected animals. As early as 5 weeks p.i. the IFN-γ test classified seven animals as positive in the high-dose group. Only four animals did not show any sensitization. In the low-dose group, none of the animals was positive at 5 weeks p.i., whereas at 11 weeks p.i., two animals were positive. Since the intradermal tuberculin test was performed at 11 weeks p.i., it is likely that this has had an influence on the IFN-γ results. This has also been demonstrated in cattle in experimental conditions (Walravens, Wellemans, Weynants, Boelaert, Debergeyck, Letesson, Huygen & Godfroid 2002). The intradermal tuberculin test could also have been the cause of the false positive test result in one of the uninfected controls at 15 weeks p.i. Ryan, Buddle & De Lisle (2000) found the IFN-γ assay to be a valuable ancillary test in cattle and that it was able to accurately predict the BTB status of an animal that was skin tested 8–28 days previously. The IFN-γ assay can be repeated at regular intervals with the advantage of only a single anaesthesia per animal. The intradermal tuberculin test on the other hand, is associated with high costs because

of the double anaesthesia as well as a time limit due to the required interval of 3 months between tests.

At the end of the study, *M. bovis* could only be isolated from six animals from the low-dose group, while five animals were negative. Four of the six animals that were positive on culture were also classified positive by the IFN-γ test at time of euthanasia. In the high-dose group, all nine animals were positive. Altogether these results show that the IFN-γ test detected BTB as early as 5 weeks p.i. in the high-dose group, as was the case in cattle. When performed at 21–22 weeks p.i., all the animals with macroscopic lesions and positive culture results also tested positive with the IFN-γ test. In this model, positive IFN-γ results correlate with the establishment of the infection in the host. All the animals in the low-dose group that were able to clear the infection, were classified negatively by the IFN-γ test at slaughter.

Throughout the study the infected buffalo calves had growth rates comparable to those of the controls. The lesions that developed after infection did not have any detectable effect on their general health or the average daily body mass gain. Similar findings have been reported in deer, where equivalent doses of virulent *M. bovis* were administered by the intratonsilar route (Griffin, Mackintosh & Buchan 1995). Mass loss has been recorded in advanced stages of BTB. (Bengis *et al.* 1996; De Vos *et al.* 2001). In buffaloes, BTB usually has a sub-clinical development and manifestations, such as poor body condition, coughing and mass loss is only found in animals with advanced disease. Our results suggest that experimental *M. bovis* infection at doses of up to 3×10^4 cfu will not affect the body condition or mass gain in young animals for at least 6 months p.i.

Because of the time frame of the experiment and the challenge route of choice, it was expected that very few animals would develop disseminated lesions. This infection model appears to be safe to work with, and effective in producing tuberculous lesions comparable with early natural infections in wild buffaloes. This model may be considered for use in further research studies where a longer time frame might allow for secondary spread of lesions. The higher infectious dose is considered the "dose of choice" for future trials since 3×10^4 cfu produced consistent and reliable infections with associated lesion development. The successful completion of the infection model will facilitate further studies of this disease problem, including evaluation of the efficacy of BCG vaccination or other vaccine candidates.

AKNOWLEDGEMENTS

The authors are grateful for the input and co-operation of the following groups of people, for without them this project would not have been possible. Field staff of the Office of the State Veterinarian for the care and feeding of the animals and daily cleaning of the boma facilities; J. Malan, M. Kruger, J. van Altena, P. Mashile and J. Muhlanga from the Game Capture team for capture, transport and weighing of the calves; veterinary technicians (Schalk van Dyk, At Decker, Johan Oosthuizen and Kenneth Muchocho); laboratory assistant (Li-Ann Small) and students (Nerina Belcher, Justin Bowers, Matthew Brownie, Jana Meyer) for their help during the sampling sessions. A special thank you to Albert Linsky (Senior Meat Inspector) and his team for the slaughtering and processing of the buffalo carcasses, Judith Kruger for statistical analysis of the data and Prof. Jacques Godfroid for extensive overview and recommendations. This project would not have been possible without the major financial contributions of the Smithsonian Institute and Shirley Sichel, in the USA.

REFERENCES

BENGIS, R.G. & RAATH, J.P. 1993. Chemical capture of the African buffalo (*Syncerus caffer*), in *The capture and care manual*, edited by Andrew A. McKenzie. Section B4: 583–597.

BENGIS, R.G., KRIEK, N.P.J., KEET, D.F., RAATH, J.P., DE VOS, V. & HUCHZERMEYER, H.F.A.K. 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer*—Sparrman) population in the Kruger National Park: a preliminary report. *Onderstepoort Journal of Veterinary Research*, 63:15–18.

BUCHAN, G.S. & GRIFFIN, J.F.T. 1990. Tuberculosis in domesticated deer (*Cervus elaphus*): A large animal model for human tuberculosis. *Journal of Comparative Pathology,* 103: 11–23.

BUDDLE, B.M., KEEN, D., THOMSON, A., JOWETT, G., MCCARTHY, A.R., HESLOP, J. & DE LISLE, G.W. 1995. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae. Research in Veterinary Science,* 59:10–16.

BUDDLE, B.M., SKINNER, M.A. & CHAMBERS, M.A. 2000. Immunological approaches to the control of tuberculosis in wildlife reservoirs. *Veterinary Immunology and Immunopathology,* 74:1–16.

BUDDLE, B.M., WARDS, B.J., ALDWELL, F.E., COLLINS, D.M. & DE LISLE, G.W. 2002. Influence of sensitization to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine,* 20:1126–1133.

CASSIDY, J.P., BRYSON, D.G., POLLOCK, J.M., EVANS, R.T., FORSTER, F. & NEILL, S.D. 1998. Early lesion formation in

cattle experimentally infected with *Mycobacterium bovis*. *Journal of Comparative Pathology,* 119:27–44.

CORNER, L.A.L., BUDDLE, B.M., PFEIFFER, D.U. & MORRIS, R.S. 2001. Aerosol vaccination of the brushtail possum (*Trichosurus vulpecula*) with bacille Calmette-Guérin: the duration of protection. *Veterinary Microbiology,* 81:181–191.

CORNER, L.A.L., BUDDLE, B.M., PFEIFFER, D.U. & MORRIS, R.S. 2002. Vaccination of the brushtail possum (*Trichosurus vulpecula*) against *Mycobacterium bovis* infection with bacille Calmette-Guérin: the response to multiple doses. *Veterinary Microbiology,* 84:327–336.

CROSS, M.L., LABES, R.E. & MACKINTOSH, C.G. 2000. Oral infection of ferrets with virulent *Mycobacterium bovis* or *Mycobacterium avium*: susceptibility, pathogenesis and immune responses. *Journal of Comparative Pathology,* 123: 15–21.

CROSS, M.L., LABES, R.E., GRIFFIN, J.F.T. & MACKINTOSH, C.G. 2000. Systemic but not intra-testinal vaccination with BCG reduces the severity of tuberculosis infection in ferrets (*Mustela furo*). *International Journal of Tuberculosis and Lung Disease,* 4:473–480.

DE VOS, V., BENGIS, R.G., KRIEK, N.P.J., MICHEL, A., KEET, D.F., RAATH, J.P. & HUCHZERMEYER, H.F.K.A. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research,* 68:119–130.

GRIFFIN, J.F.T., MACKINTOSH, C.G. & BUCHAN, G.S. 1995. Animal models of protective immunity in tuberculosis to evaluate candidate vaccines. *Trends in Microbiology,* 3:418–422.

GRIFFIN, J.F.T, MACKINTOSH, C.G., SLOBBE, L., THOMSON, A.J. & BUCHAN, G.S. 1999. Vaccine protocols to optimise the protective efficacy of BCG. *Tubercle and Lung Disease,* 79:135–143.

GRIFFIN, J.F.T., CHINN, D.N., RODGERS, C.R. & MACKINTOSH, C.G. 2001. Optimal models to evaluate the protective efficacy of tuberculosis vaccines. *Tuberculosis*, 81:133–139.

GROBLER, D.G., MICHEL, A.L., DE KLERK, L. & BENGIS, R.G. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 69:221–227.

KEET, D.F., KRIEK, N.P.J., HUCHZERMEYER, H. & BENGIS, R.G. 1994. Advanced tuberculosis in an African buffalo (*Syncerus caffer* Sparrman). *Journal of the South African Veterinary Association,* 65:79–83.

KEET, D.F., KRIEK, N.P.J., PENRITH, M.L., MICHEL, A.L. & HUCHZERMEYER, H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. *Onderstepoort Journal of Veterinary Research,* 63:239–244.

KEET, D.F., KRIEK, N.P.J., BENGIS, R.G., GROBLER, D.G. & MICHEL, A.L. 2000. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 67:115–122.

KEET, D.F., KRIEK, N.P.J., BENGIS, R.G. & MICHEL, A.L. 2001. Tuberculosis in kudus (*Tragelaphus strepsiceros*) in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 68:225–230.

MACKINTOSH, C.G., WALDRUP, K., LABES, R., BUCHAN, G. & GRIFFIN, J.F.T. 1995. Intra-tonsil inoculation: an experimental model for tuberculosis in deer. *Tuberculosis in wildlife and domestic animals.* Dunedin: Otago University Press.

MICHEL, A.L., NEL, M., COOPER, D. & MOROBANE, R.N. 2000. Field evaluation of a modified "gamma interferon" assay in African buffalo (*Syncerus caffer*) and cattle in South Africa. *Proceedings of the 3rd International Conference on Mycobacterium bovis,* 14–16 August 2000, Cambridge, United Kingdom.

MICHEL, A.L. 2002. Implications of tuberculosis in African wildlife and livestock. *Annals of the New York Academy of Sciences,* 969:251–255.

PALMER, M.V., WHIPPLE, D.L., RHYAN, J.C., BOLIN, C.A. & SAARI, D.A. 1999. Granuloma development in cattle after intratonsilar inoculation with *Mycobacterium bovis*. *American Journal of Veterinary Research*, 60:310–1315.

RODWELL, T.C., KRIEK, N.P., BENGIS, R.G., WHYTE, I.J., VILJOEN, P.C., DE VOS, V. & BOYCE, W.M. 2001. Prevalence of bovine tuberculosis in African buffalo at Kruger National Park. *Journal of Wildlife Diseases,* 37:258–264.

RYAN, T.J., BUDDLE, B.M. & DE LISLE, G.W. 2000. An evaluation of the gamma interferon test for detecting bovine tuberculosis in cattle 8 to 28 days after tuberculin skin testing. *Research in Veterinary Science,* 69:57–61.

TANNER, M. & MICHEL, A.L. 1999. Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park. *Onderstepoort Journal of Veterinary Research,* 66:185–190.

VORDERMEIER, H.M., CHAMBERS, M.A., COCKLE, P.J., WHELAN, A.O., SIMMONS, J. & HEWINSON, R.G. 2002. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infection and Immunity,* 70:3026–3032.

WALRAVENS, K., WELLEMANS, V., WEYNANTS, V., BOELAERT, F., DEBERGEYCK, V., LETESSON, J-J., HUYGEN, K. & GODFROID, J. 2002. Analysis of the antigen-specific IFN-γ producing T-cell subsets in cattle experimentally infected with *Mycobacterium bovis*. *Veterinary Immunology and Immunopathology,* 84:29–41.