Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park

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ABSTRACT

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

The survival time of *Mycobacterium bovis* in the natural habitat of infected free ranging wildlife was investigated. Seven different experimental sites near Skukuza in the Kruger National Park, South Africa, were selected to expose macroscopically affected lung or lymph node tissue of African buffalo *(Syncerus caffer)* origin and spiked faecal specimens for various lengths of time over a 1-year period. *Mycobacterium bovis* could be isolated for a maximum period of 6 weeks from tissue specimens and 4 weeks from faeces. The longest survival of *M. bovis* in both specimen types was observed in winter and under moist conditions. Surprisingly, the survival time of *M. bovis* in buried specimens seemed greatly reduced to a maximum of 5 days.

Keywords: African buffalo, environmental conditions, Kruger National Park, *Mycobacterium bovis*, viability

INTRODUCTION

Tuberculosis is an exotic disease in southern Africa, brought in by English and Dutch cattle herds during colonialization in the 19th century (Webb 1936; Myers & Steele1969). The first case of tuberculosis in cattle was reported by Hutcheon 1880 (Henning 1956). In South African wildlife, Paine & Martinaglia (1928) reported the first infections caused by *Mycobacterium bovis* in free-living greater kudu (*Tragelaphus strepsiceros*) and common duiker (*Sylvicapra grimmia*). In the Kruger National Park bovine tuberculosis was first diagnosed in July 1990 in an African buffalo (*Syncerus caffer*) in (Bengis, Kriek, Keet, Raath, de Vos & Huchzermeyer 1996). Circumstantial evidence supported by DNA fingerprinting indicates that *M. bovis* infection was introduced into the KNP via an infected cattle herd on its southern border some 40 years ago (unpublished data). Today, tuberculosis occurs widely in the southern region of the KNP and is spreading northwards, having nearly reached the northern border (Keet, Kriek, Huchzermeyer & Bengis 1994). In some buffalo herds the prevalence of tuberculosis is as high as 90% (Keet, personal communication). Since late 1995 it has become evident that the infection has spilled over into other species such as lion (*Panthera leo*), cheetah (*Acinonyx jubatus*), chacma baboon (*Papio ursinus*), greater kudu (*Tragelaphus strepsiceros*) (Keet, Kriek, Penrith & Huchzermeyer 1996) and leopard *(Panthera pardus)* (Bengis, personal communication 1998). It became clear that a strategic plan to contain, control and eventually eradicate tuberculosis in the KNP was needed. In order to ensure the success of such a plan the risk of reinfection of "clean" herds from a contaminated environment must be minimized. Therefore it is essential to gather scientific data

Accepted for publication 6 July 1999—Editor

which indicate for how long *M. bovis* can retain its infectivity outside the host under different environmental conditions in the Kruger National Park. Morris, Pfeiffer & Jackson (1994) concluded from an extensive literature review that the maintenance of infectivity of *M. bovis* outside the host is generally substantially below the period for which bacilli can be recovered by culture isolation. They also stated that mycobacteria on naturally contaminated material tend to die more quickly than do those on experimentally infected but otherwise equivalent material. Subsequently this research project was designed to investigate the maximum survival time of *M. bovis* in tissue and faecal specimens which can, under natural conditions, serve as potential sources of *M. bovis* infection to free-ranging wildlife. The experiment was carried out at 3-monthly intervals to account for seasonal climatic variations over the period of 1 year. Maximum lengths of time for which *M. bovis* could be recovered from the specimens were determined.

MATERIALS AND METHODS

Specimens

Naturally infected lung or lymph node tissues from tuberculous buffalo *(Syncerus caffer)*, from the southern part of KNP and experimentally infected faeces from extensively kept cattle were exposed to the various environmental conditions. In a laboratory at Skukuza in the KNP lungs or lymph nodes containing lesions caused by *M. bovis* infection were cut into pieces weighing 80–100 g and approximately 40–50 g of cattle faeces were spiked with *M. bovis* (ATCC 19210) respectively with 8×10^7 organisms.

Experimental sites

Both sample types were exposed to the different environmental conditions within fenced bomas near Skukuza in the southern KNP. Seven experimental sites for exposure of the specimens were selected (cf. Table 1). Four sites were at ground level with two being dry soil and the other two permanently moist soil. Two artificial water holes (each 1,5 x 1,5 m x 0,2 m) were created in the boma for this purpose which were maintained by a running water tap and which ensured that the specimens in the cages were surrounded by water during the entire duration of the experiment. Each site was either exposed to full sun or full shade. Sunny and shady conditions were also selected for two sets of specimens placed at a height of approximately 1,5–2,0 m. An additional set of samples was buried in moist soil 20 cm below the surface. Specimens were recovered after time intervals ranging from two days to eight weeks and stored at –20 °C until transferred to Onderstepoort Veterinary Institute, which is situated near Pretoria.

TABLE 1 Experimental sites

	Dry soil	Moist soil	Tree height
Shade	•	•	•
Sun	•	•	•
Subterranean		•	

TABLE 2 Average soil temperatures[a] at the experimental sites

Depth	Jul.	Aug.	Sep.	Nov.	Dec.	Feb.	Apr.
10 cm	23,5	27,6	30,5	33,3	32,7	35,6	32,3
20 cm	19,9	23,2	26,5	29,9	30,2	31,6	28,6
30 cm	18,9	21,8	24,3	27,5	27,9	29,7	27,3

[a] Average soil temperatures as measured at 14:00

Exposure of specimens

A set of samples consisting of 20 plastic cups (100 mℓ), 10 containing pieces of tissue and 10 containing spiked faeces were prepared for each experimental site. Each set was arranged on 3 polystyrene trays (28 x 20 cm). The trays were placed inside a wire cage (70 x 30 cm) which was wrapped in mosquito netting to provide protection against small predators. The wire cages were each deposited in a larger, sturdy metal cage to prevent scavenging of the specimens by larger predators. At the dry sites the specimen trays were placed on bricks inside a metal tray filled with a mixture of oil and water for additional protection from ants. An additional set of samples was immediately taken back to the laboratory without exposure to serve as control.

Intervals of exposure of specimens

During winter and spring specimens were exposed for 5 d, 2 weeks, 4 weeks, 6 weeks and 8 weeks, respectively. In the following summer and autumn the periods of exposure were shortened to 2 d, 5 d, 8 d, 2 weeks and 3 weeks, respectively. The buried samples were exposed for periods of time varying from 5 d to 18 weeks. With each collection of specimens duplicate sets of tissue and faeces were removed for culture from each experimental site.

Bacterial isolation

Initially tissue specimens were processed using 4 % NaOH or 2 % HCl for decontamination and distilled H_2O for neutralization ((Bengis *et al.* 1996). Faeces were decontaminated with 4 % NaOH, whereas neutralization was carried out with 10 % H_3PO_4. Due to a high rate of contamination both methods were subsequently modified and 4 % NaOH for decontamination and 5 % oxalic

acid for neutralization were used. After inoculation onto Loewenstein-Jensen egg medium containing either glycerine or pyruvate, media slants were incubated at 37 °C until growth was observed or for a maximum period of 10 weeks. Subsequent subcultures of the isolates were identified by polymerase chain reaction (Keet *et al.* 1996).

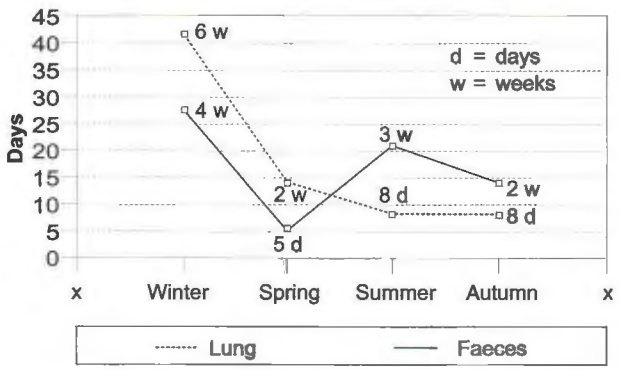

FIG. 1 Survival time of *M. bovis* in the Kruger National Park

Collection of insects and worms inhabiting the specimens

During the spring and summer experiments the majority of larvae of flies, beetles, centipedes and worms found to be inhabiting the specimens were collected and processed for culture isolation.

Macroclimatic data

Minimum and maximum temperature, humidity and rainfall data were recorded at the weather station at Skukuza, located approximately 200 m from the experimental sites. The soil temperature in various depths was repeatedly measured during each season.

RESULTS

Bacterial isolation

The longest periods of survival of *M. bovis* in infected specimens were observed during winter. Viable *M. bovis* could be recovered for up to 6 weeks from lung

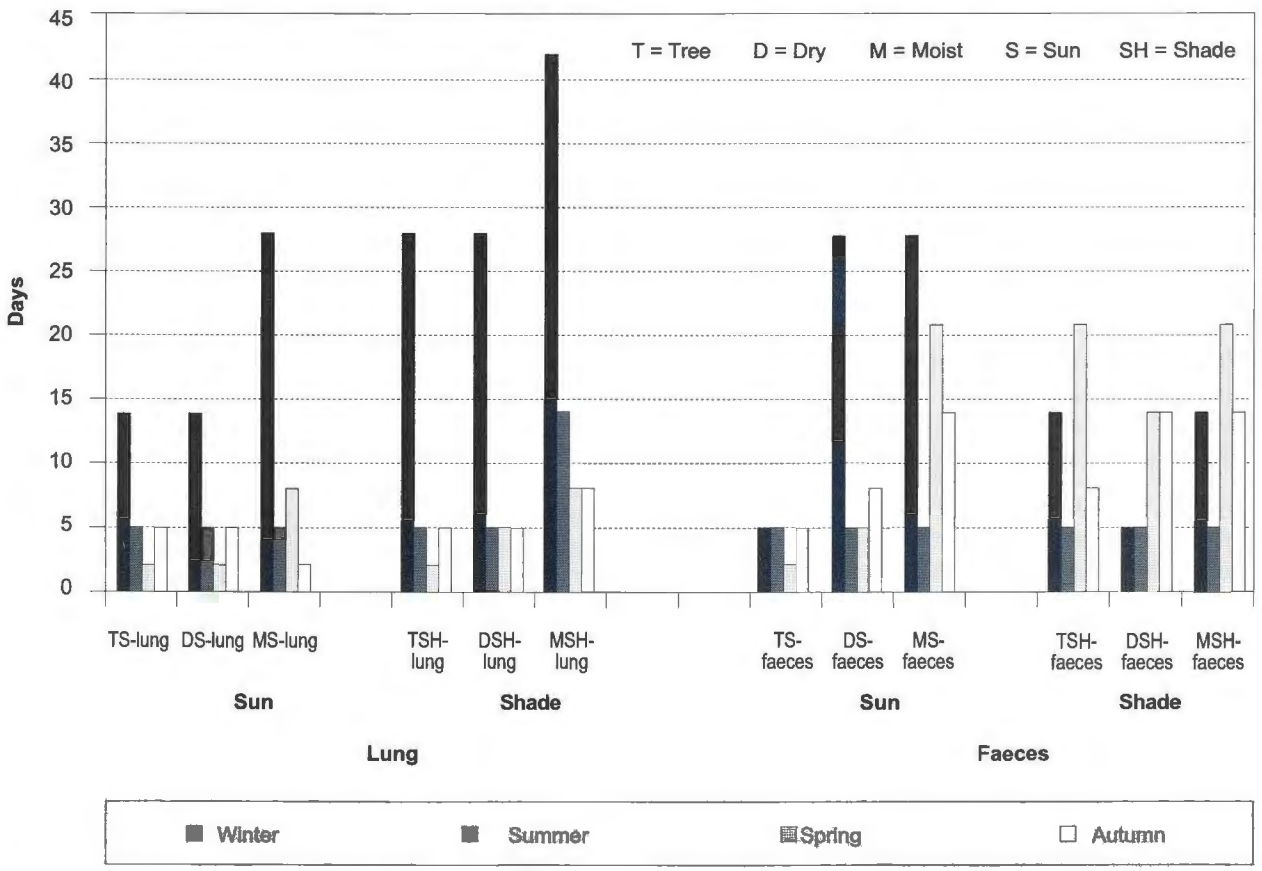

FIG. 2 Survival times of *M. bovis* under various conditions

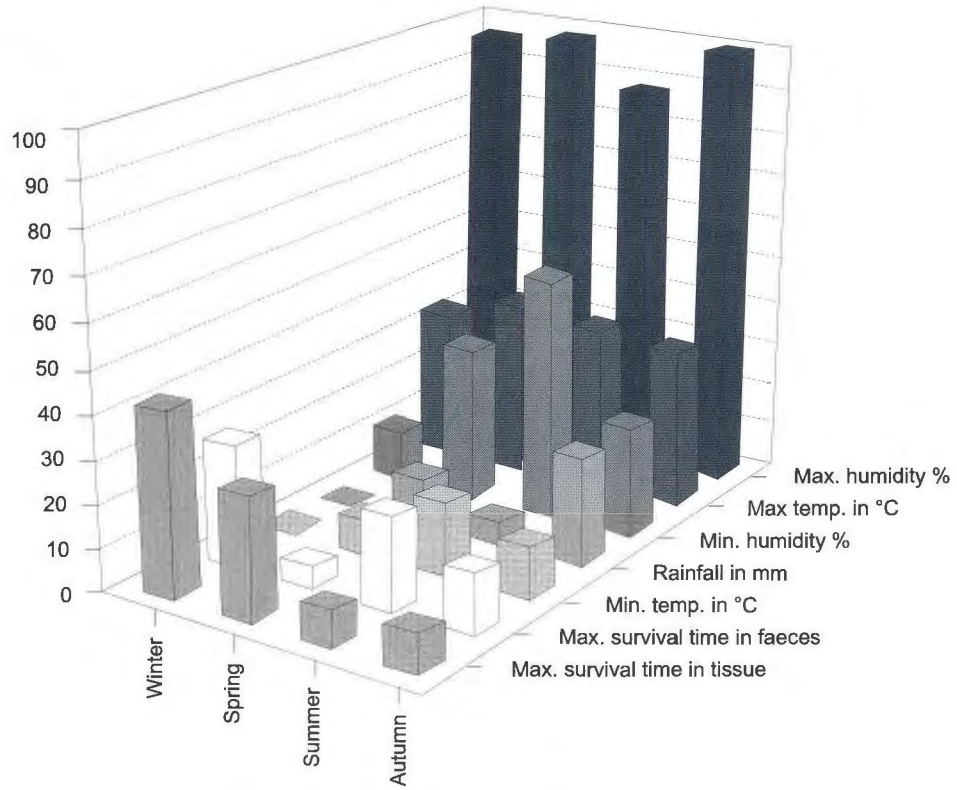

FIG. 3 Meterelogic data in comparison with maximum survival times

tissue and up to 4 weeks from faeces. Its viability in tissue specimens (Fig. 1) showed a five-fold decrease from winter to summer, while survival times of *M. bovis* in faecal samples were found to vary only by 1 week throughout the year. Subterranean specimens were found to be negative for *M. bovis* at all times except for spring when a successful isolation was made from specimens buried for 5 d.

With regard to the influence of microclimatic conditions on the survival of *M. bovis* in both specimen types, isolations were more successful from specimens exposed to moist conditions. During the spring and summer period a combination of moisture and shade generally provided the best support to *M. bovis*. In contrast, the slightly longer survival time in faeces during winter was found at moist but sunny experimental sites. Due to insufficient protection against insects in most specimens at shady experimental sites larvae of flies were found. Some of them were also infested by worms, centipedes and beetles. Infestations increased with the duration of exposure. Due to this intensity of contamination, in several cases only the hardened outer surface of the lung tissue was left over after exposure. All the worms, larvae and adult insects found in the specimens were submitted to culture examination but found negative for *M. bovis*.

Macroclimatic data

Metereologic data over the entire period of our investigation are summarized in Fig. 3. These include minimum and maximum temperatures, minimum and maximum humidities as well as rainfall as recorded at the weather station at Skukuza. The soil temperatures measured at the experimental sites in depths varying from 10–30 cm ranged between 18,9 °C in winter and 35,6 °C in summer (Table 2).

DISCUSSION

This study represents the first of its kind carried out using naturally infected animal tissue in the infected ecosystem under various macro- and microclimatic conditions in southern Africa. Lung or lymph node specimens exposed in winter were found to harbour viable *M. bovis* for up to 6 weeks, whereas in the other seasons the survival time of *M. bovis* varied from 5–14 d (Fig. 1). Previous investigations of the survival time of *M. bovis* in wildlife were mainly carried out on possum and badger carcases in New Zealand (Pfeiffer & Morris 1991) and England, (Little, Naylor & Wilesmith 1982, Anon 1979, O'Reilly & Daborn 1995), respectively. The survival times in these studies ranged from 2–4 weeks but the possible impact of seasonal variations and different

environments remained unexplored. In spiked bovine faecal samples exposed along with the naturally infected buffalo tissue we found that *M. bovis* could survive for up to 4 weeks which is in conformity with the reports of Duffield & Young (1985) and Anon (1979). The latter further observed that naturally infected badger faeces did not contain viable *M. bovis* for longer than 1 week supporting the conclusion of Morris *et al.* 1994 that the length of survival is shorter in naturally infected than in spiked material.

With regard to the identification of factors affecting the survival time of *M. bovis* we observed that moisture and temperature mainly determined the length of time for which *M. bovis* remained viable in our study. Throughout the experiment the longest surviving *M. bovis* isolates in each season were recovered from specimens at moist and shady sites suggesting that these conditions are able to support mycobacterial survival better than dry and sunny environments. This implies that under natural conditions moisture is the most important limiting factor to the viability of *M. bovis*. In nature, moisture is provided either by macroclimatic factors such as rainfall and humidity or within the microenvironment by rivers and water holes. Under the conditions of the artificial water holes *M. bovis* survived considerably longer than in the dry soils. The higher rainfall during the summer period, however, could not enhance the survival of *M. bovis* at the dry experimental sites (Fig. 3). No correlation between the seasonal humidity pattern and the maximum survival time of *M. bovis* could be found and we conclude that these macroclimatic factors had no influence on the viability of *M. bovis*.

The fact that the maximum survival time was recorded in winter indicates that temperature might further play a key role. While the seasonal fluctuation of the maximum temperature was insignificant (Fig. 3), the minimum temperature in winter was 16,3 °C below the minimum temperature in summer suggesting that the longer survival of *M. bovis* could be ascribed to the low minimum temperature. Morris *et al.* (1994) explained this phenomenon as follows. Low minimum temperatures delay the warming up of the specimens during the day and therefore the loss of water is slowed down. In the work of Duffield & Young (1985) both these criteria prevail as enhancers of the survival of *M. bovis* by stating that moist soil favoured the survival of *M. bovis* under high temperatures (43 °C) but not so in the cool laboratory environment. We speculate that the drying and hardening of the specimen surface furthermore helped to minimize evaporation because a number of isolates were made from specimens of which only the hard surface was left for culture.

Ultraviolet light was not found to have a limiting effect on the viability of *M. bovis* in the specimens examined. In contrast, we recovered the longest surviving *M. bovis* isolates in faeces from specimens exposed to sunny but moist conditions (Fig. 2). Only in the absence of water the sunlight caused extensive desiccation hence attributing to a reduced viability of *M. bovis*.

Apart from the macro- and microclimate a number of other external factors can have a negative effect on the survival time of *M. bovis*. In some of the studies on badgers, the carcases left in accessible locations were scavenged completely within 2–3 d. In carcases not scavenged the activity of other bacteria, protozoa and fungi that normally contribute to the breakdown of faeces or tissues, also appears to destroy tubercle bacilli. The natural decomposition of a carcass as a whole is believed to destroy *M. bovis* (Anon 1979). Scavenging of the specimens was prevented in our experiment by the use of large, sturdy cages but the severe infestation of the tissue with small predators such as adult flies and larvae of flies, beetles, centipedes and worms led to the loss of a considerable amount of infected tissue. Likewise Williams & Hoy (1930) found that the survival time of *M. bovis* in cattle faeces increased from 2–6 months after protection from insects and worms during the northern European summer. They were, however, unable to culture *M. bovis* from soil of an area grazed by a known *M. bovis* excreting cow. Whether ingestion by arthropods and worms in fact leads to the destruction of *M. bovis* or may, on the other hand, provide enhanced chances for survival can presently not be answered. Although none of the arthropods or worms was found to be culture positive in this study their possible involvement in the epidemiology of tuberculosis cannot be excluded without further investigations.

It was also found that specimens became increasingly with time contaminated with competitive environmental bacteria. As reported elsewhere they are able to utilize nutrients which supply the basic requirements for the survival of slow growing mycobacteria and thus negatively influence the survival time of *M. bovis* (Passini 1926, Woldrich & Singer 1931, Sewall & De Savitsch 1937). Anon (1979), for example, reported on the storage of artificially infected soil together with the original *M. bovis* culture at ambient temperature. After 7 months only the original culture contained viable *M. bovis* still virulent for guinea pigs. We could only isolate *M. bovis* from specimens buried in moist soil not longer than 5 d. Bacterial antagonism and desiccation due to high soil temperatures might be accountable for the failure to recover *M. bovis* thereafter. Similarly O'Reilly & Daborn (1995) failed to isolate *M. bovis* from three buried badger carcases after 2, 3 and 6 weeks, respectively. We therefore believe that *M. bovis* in contaminated soil is not likely to survive longer than in naturally infected tissue.

In summary, the results presented here demonstrated differences in the ability of *M. bovis* to survive outside the host which depend on both the environment and the temperature with the moisture being the most critical requirement. With a range of limiting factors such as withdrawal of water, predators and competitive bacteria the survival time of *M. bovis* under natural circumstances can in most cases be considered shorter than under the partially optimized conditions applied during this experiment. As mentioned earlier it is further believed that the infectivity of *M. bovis* in environmental sources for susceptible species like cattle, and probably also wildlife, is below the survival time of *M. bovis* as determined by artificial isolation.

ACKNOWLEDGEMENTS

The authors thank the staff of Veterinary Services at Skukuza for providing funds and expertise in collecting infected tissue from buffalo and for assistance in collecting the exposed samples at the experimental sites. We also thank Mrs B. Bengis for providing metereologic data for the entire period of the investigation. We are indebted to the staff of the tuberculosis laboratory of the Onderstepoort Veterinary Institute, especially Mr S.J. Waterson and Mrs S.J. Smith, for competent technical assistance.

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