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Bovine Tuberculosis in Brushtail Possums
(*Trichosurus vulpecula*):
Studies on Vaccination, Experimental Infection, and
Disease Transmission.

A thesis presented
in partial fulfilment of the requirements for the degree of
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Abstract:

The objectives of the research program were to obtain a better understanding of BCG as a tuberculosis vaccine in possums, and assess its potential as a tool for controlling tuberculosis in wild possum populations. A series of vaccination and challenge experiments were conducted, as well as studies on alternative experimental infection procedures. The program included two field studies, one on the epidemiology of tuberculosis in a population of possums regenerating after localised possum eradication, and the other examined the efficacy of BCG vaccine in a wild population in which tuberculosis was endemic.

The first experiments confirmed the earlier published findings that BCG delivered as an intranasal aerosol induced a protective response. The protective response was found to be present 12 months after vaccination and therefore of sufficient longevity to make vaccination a practical control tool. A second study demonstrated that revaccination of possums enhanced protection and a third showed that conjunctival vaccination was as effective as intranasal aerosol. These findings supported the development of a possum activated self-vaccinator that would deliver vaccine as an aerosol. In delivering the spray to both the external nares and the eyes a simple and cheap device could be designed to efficiently vaccinate wild possums.

The intratracheal experimental infection procedure used in the vaccination and challenge experiments was not entirely suitable for our purposes. Although it provided an assured level of exposure and repeatable results, all infected possums developed fulminant, rapidly progressive disease, irrespective of the vaccination regime used. Two alternative methods of challenge were examined; the conjunctival route of infection, and natural transmission between experimentally infected possums and susceptible in-contact possums. Conjunctival infection was shown to be a reliable procedure for infecting possums, with the disease that resulted from infection having many of the cardinal features of natural tuberculosis in wild possums. Infection following conjunctival inoculation progressed slowly and may be suitable for studying pseudo-vertical transmission and the efficacy of post-infection vaccination.

In studies with captive possums there was little or no transmission of infection between experimentally infected possums and susceptible in-contact possums in the same pen when

the experimentally infected animals were selected at random. However, when possums with high levels of social interaction were experimentally infected there was a significant increase in transmission rates. In addition, the possums that became infected by transmission were more socially active than those that remained free of infection.

Two aspects of the pathogenesis of tuberculosis in possums were clarified during the experimental infection and natural transmission studies. The duration of preclinical infection, impossible to determine accurately in longitudinal studies on wild possums, was found to range from 6 - 20 weeks. Secondly, the pre-eminence of the aerosol route in naturally transmitted tuberculosis was confirmed.

After eradication of possums from a 36 ha site, tuberculosis reappeared within four months. Re-emergence of infection on the site was due to immigration of infected possums, not to the survival of *M. bovis* in the environment. Each of the four restriction endonuclease analysis (REA) types of *M. bovis* that caused disease in the possum population showed a different temporal and spatial pattern.

BCG vaccine had high efficacy in a wild possum population. Over 2 years, 300 possums were recruited to a study of BCG vaccination. Approximately 50% of the possums were vaccinated, where each possum was vaccinated using both intranasal aerosol and conjunctival instillation. There were significantly more cases of tuberculosis in unvaccinated possums than in vaccinates, with a relative risk of tuberculosis in unvaccinated possums of 3.21. The vaccine efficacy was 69%. The most important question relating to BCG vaccine that remains to be addressed is the ability of vaccination to control tuberculosis in possum populations.

This research has demonstrated that BCG vaccine provided protection against *M. bovis* infection in both captive and wild possums. Future research should be directed towards developing delivery systems for vaccinating wild possums and strategies for vaccine use in wild tuberculous possum populations.

Acknowledgements

“A Chinese fable tells of a young man discovering a sage at the village well. The old man was lowering a wooden bucket on a rope and pulling the water up slowly, hand over hand. The youth disappeared and returned with a pulley. He approached the old man and showed him how the device worked. “See, you put your rope around the wheel and draw up the water by cranking the handle”. The old man resisted. “If I use a device like this, my mind will think itself clever. With a cunning mind I will no longer put my heart into what I am doing. Soon my wrists alone will do the work. If my heart and whole body are not in my work, my work will become joyless. When my work is joyless, how do you think the water will taste.”

Kornfield, J. 2000, “After the Ecstasy, the Laundry”, Random House, Sydney, p. 189.

For me there are few greater joys than learning. I cannot imagine a more satisfying vocation than research. No greater responsibility could be asked of me than to conduct research openly, honestly, diligently and ethically. However, life is for living, and is to be lived here and now. Living is to be joyful and not to be wasted, life is too valuable to be “put off” until the PhD is finished. The Buddha advised that we live intentionally, live in the moment. That is what I intended to do. There was no greater sadness for me than when my PhD studies became a burden. When that happened my research suffered, the quality became poor because I was not attending to the work with all my mind and all my heart. My research, my learning, my life became joyless. But with the help of my friends and fellow students I rebounded after a short time. During my time as a student I have conducted the best research in my career although it has not been my most productive time.

Many people have helped me during my studies and I am indebted to each and every one. My fellow students have been an inspiration and support, adding their humour, wisdom and friendship. I want to acknowledge especially Carola Sauter-Louis, Solis Norton and my daughter René. Deb McCrae listened to my grumbles, pleas and joys and went beyond her role as the EpiCentre administrator. There were numerous others who helped especially a stream of local and foreign students, and a number of technicians and administration staff. I want to acknowledge the support, help and guidance of the academic staff outside of the EpiCentre, in the “Vet Tower”, especially that of Professors Maurice Alley, WAG (Tony) Charleston, Colin Wilks, and Dr Stan Fenwick. There were a number

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My time at Massey University has been anything but plain sailing. When I arrived I was in the process of divorce and I had resigned from my position at Commonwealth Scientific and Industrial Research Organization (CSIRO), a position that I had held for 23 years. I had left behind two daughters, extended family, friends, my whole support network. Settling down in New Zealand was difficult. At CSIRO I had been a member of several highly productive, multidisciplinary research teams and in the EpiCentre I felt isolated, and at home I was alone.

In November of 1996 I met Laurie Lawler, my wife, and my personal world once again took on some joy. Laurie has a wisdom that is unique in my experience, a wisdom based on intuition and borne of experience. She is a very intelligent person engendered with love, compassion and a level of common-sense that is very uncommon. Being supported, nurtured and loved by Laurie enabled me to continue my search for self-awareness, to understand myself, my reactions, and to continue my academic studies when I was sorely tempted to “pack it all in”. More than to anyone else I am indebted to her and to her I dedicate this work.

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Photographer: Sam Beckett

SECTION A.

Introduction.

Introduction

The thesis is set out in 5 sections: an introduction, 3 sections describing the research with each focusing on a single theme, and a general discussion of the research findings.

Section A: The Introduction is a brief review of bovine tuberculosis in New Zealand, tuberculosis in possums, the role of the possum in the epidemiology of tuberculosis, the history of BCG vaccination in possums and BCG in other species and human.

Section B contains three chapters that describe studies on vaccination of captive possums against tuberculosis: the duration of protection, the effect of multiple doses of vaccine, and the efficacy of the conjunctival route of vaccination.

Section C also contains three chapters and these address experimental tuberculosis in captive possums: a study of the social organisation of captive possum colonies, natural transmission of tuberculosis within captive colonies and experimental infection of possums using the conjunctival route of administration.

Section D describes two studies in a wild possum population: the epidemiology of tuberculosis in a possum population as it re-emerged after localised possum eradication, and the efficacy of BCG vaccination in a wild possum population.

The final Section E is a general discussion of the research findings and their broader implications.

At the beginning of each section is a brief description of the theme of the section and how the chapters in the section relate to each other. Each chapter is the manuscript of a paper published in or submitted to a peer reviewed journal. Each chapter is written in a style to suit the readership of a particular journal and format of the manuscript is that required by the journal.

The list of references used in the Introduction and in the General Discussion are found at the end of the thesis, and the references used in each of the research chapters are to be found at the end of each chapter. Pages, figures and tables have been numbered sequentially throughout the thesis.

Chapter 1

Perspective On Tuberculosis Vaccination Of Wild Possums

Introduction

The epidemiology of bovine tuberculosis, that is, infection with *Mycobacterium bovis*, in New Zealand livestock and wild animals has been the subject of many studies at the EpiCentre, Massey University. The research commenced in 1989, with a focus the epidemiology of the disease in the brushtail possum (*Trichosurus vulpecula*) (Pfeiffer, 1994; Jackson, 1995), the interrelationship of tuberculosis in possums and other wild animal species (Lugton, 1996), the biology and interaction of possums and livestock (Paterson, 1993), and the control of tuberculosis in possums and livestock (McKenzie, 2000; Sauter-Louis, 2001). The fieldwork was undertaken at Castlepoint in the Wairarapa, and at a range of other sites in the country where tuberculous wildlife existed. These studies have elucidated the part played by each animal species in the epidemiology as well as the role of environmental contamination and the elucidation of transmission pathways. They have provided a clear picture of the temporal and spatial clustering of the disease, an important component in the design and evaluation of control programs at both the regional and individual farms levels. The potential integration of vaccination into control strategies provided the impetus for the research described in this thesis.

Overall, the above theses have thoroughly reviewed the epidemiology, immunology and pathogenesis of *M. bovis* infection in animals. I have focused in the Introduction, on vaccination of possums, and aspects of *M. bovis* infection in domestic and wild animals in New Zealand, necessary to fully understand the scope of the General Discussion. The literature relevant to each of the specific areas of study is reviewed in the introduction to each of the research chapters.

In 1993, independent evaluations of vaccination concluded that the best prospects for achieving gains in tuberculosis control lay in vaccinating wildlife, possums in particular, rather than in vaccinating domestic stock (Morris et al., 1993). It was considered that vaccinating possums would be more effective than vaccinating livestock because it was believed the efficacy of the vaccine for livestock would have to be high to prevent infection. In addition, vaccinating wild animals would be less likely to lead to international trading difficulties. A World Health Organization Expert Group on Animal Tuberculosis Vaccines recommended that priority should be given to the development and evaluation of vaccines for use in wildlife in those countries which had a wildlife vector problem (WHO,

1995). They also concluded that the avirulent strain of *M. bovis*, strain bacille Calmette-Guérin (BCG) offered by far the best option for a first-generation vaccine for use in animals.

Bovine tuberculosis puts at risk New Zealand's international trade in dairy, beef and deer products. Tuberculosis in possums is the principal reason for the persistence of the disease in New Zealand (Coleman and Livingstone, 2000). Possums are not just an economic threat to New Zealand's livestock by harbouring and spreading bovine tuberculosis, but are seen as a pest for other reasons. Possums feed on trees, shrubs and pasture, they damage native and exotic forests, and commercial crops (Butcher, 2000). Through their selective browsing of native trees they are modifying native ecosystems, and by predation are interfering with native birds, including some species threatened with extinction. There were an estimated 60 – 70 million possums in New Zealand at the date of the most recent assessment (Cowan, 1991).

The brushtail possum was introduced into New Zealand from south-eastern Australia. Releases began in the mid-1800s and continued until the early 1900s. Their liberation, in numerous sites on the two main islands, was undertaken to provide the basis of a fur industry. Until the 1940s, the possum was protected from indiscriminate hunting to maintain the population (Pracy 1962).

Possums have been a very successful coloniser. They have adapted to extremes of latitude, being found from Northland to Stewart Island and from sea level to subalpine slopes, from dense temperate rain forest to open, tussock grass lands (Cowan and Clout, 2000). To control possums and tuberculosis in the possum populations, it is necessary to understand not only the epidemiology of the disease in possums but also their population dynamics and behaviour.

Bovine Tuberculosis in Animals

Cattle are the natural host of *M. bovis* (Thorne and Morris, 1983). The agents of mammalian tuberculosis are members of the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum*. Bovine tuberculosis is found worldwide and is a disease of economic importance in both developed and developing nations. *M. bovis* is

infective to all warm-blooded animals including humans (Pritchard, 1988) and most animal tuberculosis is due to *M. bovis* (Cousins and Dawson 1999; Roberts et al., 1999). It has the widest host range of all known pathogens (O'Reilly and Daborn, 1995; Thorne and Morris, 1983).

M. bovis is a zoonosis involving transmission from domestic and wild animal reservoirs to humans. An undetermined number of cases of tuberculosis in humans, especially in developing countries, are due to *M. bovis* (Cosivi, 1998). There is an increased risk of human tuberculosis, including infection with *M. bovis*, in people infected with the human immuno-deficiency virus (HIV), and especially those with clinical acquired immuno-deficiency syndrome (AIDS) (van den Broek et al., 1993; O'Reilly and Daborn, 1995).

Because of the risk to humans many developed countries have undertaken to eradicate bovine tuberculosis from domestic cattle and these have been successful in some instances, for example, Australia, Canada, Denmark and Sweden (Clifton-Hadley and Wilesmith, 1995). However, where there is uncontrolled transmission of tuberculosis between domestic and wild animals, eradication of tuberculosis from domestic animals is impracticable, if not impossible.

Bovine Tuberculosis in Wild Animals – Global perspective

Wildlife reservoirs of *M. bovis* have been found in several countries. They have been identified, for example, in bison and whitetail deer in North America (Tessaro, 1986; Schmitt et al., 1997), badgers in the UK and Ireland (Hughes et al., 1996), feral Asian water buffalo and feral pigs in Australia (Cousins and Corner 1998; Corner et al., 1981), African buffalo in South Africa (Keets, 2000) and brushtail possum and feral deer in New Zealand (Morris and Pfeiffer, 1995). Culling of the infected populations has been the preferred means of controlling tuberculosis in wildlife. Infection in feral buffalo in Australia was eradicated by culling the buffalo population. With the eradication of tuberculosis from domestic cattle and the eradication of wild buffalo, the disease in feral pigs disappeared as the feral pig was a dead end host (Cousins and Corner, 1998). Culling of infected wildlife populations is an unpopular option where the species has a high conservation value, as with the bison, African buffalo and badgers, or has high economic value, as with the whitetail deer. In the United Kingdom and Ireland a major limitation in

controlling badger populations for tuberculosis control are conservation, political and emotional issues surrounding the badger. In the case of the badger, culling of disease sets has been undertaken but the effectiveness has been greatly debated (Krebs, 1997). In North America both conservation and economic issues limit the available options for the control of the wildlife vectors.

In New Zealand, the principal wildlife reservoir of tuberculosis is the brushtail possum but tuberculosis is also widely distributed in feral deer and ferret populations. The role of the ferret in the epidemiology of tuberculosis is unclear (Morris and Pfeiffer 1995) and is discussed in more detail below. All three species are regarded as pests. The possum is both an ecological and economic pest, ferrets are primarily an ecological pest, and feral deer are both an ecological pest and an economic resource.

Culling of wildlife vectors in New Zealand is not without limitations. Apart from the expense of the control program there are concerns about the use of toxins for population control of possums, deer and ferrets. Control programs for possums and deer have significant positive ecological benefits but there are concerns about the adverse effects of toxins on non-target species. Some of the proposed new control methods, for example, control using infectious agents, generate both animal welfare concerns and fears of risk to the human population.

Culling of infected wildlife species is the most frequently used strategy for the control of tuberculosis in wild animals. Where the infected species has high value, alternative strategies for control will have to be developed. This will be especially true where culling alone is uneconomic. Despite intensive efforts to cull infected possum populations to levels below that estimated necessary to prevent transmission (Roberts and Kao, 1997), tuberculosis has persisted in the culled populations. The most promising alternative option in the short term is vaccination and in the longer term vaccination and biological control.

Bovine Tuberculosis in New Zealand

In most countries bovine tuberculosis has been a serious detriment to the health of cattle and a risk to the human population. As a consequence, most developed countries,

including New Zealand, have embarked upon programs to eradicate bovine tuberculosis from domestic livestock.

The history of bovine tuberculosis control in New Zealand is similar to that in other developed countries. The transmission of infection from dairy cattle to humans through consumption of fresh milk and dairy products made from raw milk, lead to disease in children. This in turn lead to the introduction of pasteurisation of milk and the desire, through voluntary and later compulsory schemes, to free dairy cattle from tuberculosis. Control of bovine tuberculosis in cattle in New Zealand was initially achieved, in the absence of possum tuberculosis, by maintaining strict control of the movement of cattle from infected herds, regular tuberculin test surveillance of all herds and abattoir monitoring of slaughtered cattle (Tweddle and Livingstone, 1994). The prospect for the successful eradication of bovine tuberculosis from New Zealand looked very good throughout the 1960s and 1970s (Tweddle and Livingstone, 1994). National prevalence was continuing to drop and it was optimistically expected that bovine tuberculosis would be eradicated. It was not until the prevalence was low, through the 1970s, that the presence of wildlife reservoirs became apparent. The significance of these reservoirs and there potential impact on tuberculosis control was not immediately comprehended.

The New Zealand bovine tuberculosis control strategy was, and still is, based on a test and slaughter program. Such programs have proved successful in a number of countries, notably Australia, North America and Western Europe. However, in New Zealand while domestic livestock are continually being infected from wildlife reservoirs, national eradication is not possible.

The designers of the earlier bovine tuberculosis eradication schemes in New Zealand were optimistic that complete eradication was achievable. In the design of the original campaign the only animal species involved were cattle. They understood the epidemiology to involve a simple transmission pathway, from infected cattle to susceptible cattle. Detecting and removing infected animals would interrupt the transmission pathway and lead to eradication. The significance of the 1970 report of *M. bovis* infection in wild possums and the possibility of a wild animal reservoir of *M. bovis* was not immediately appreciated (Davidson, 1976). The information did not have any immediate impact on the campaign or the expectations for eradication. Localised possum control, associated with

known foci of infection, was undertaken. It was not until the prevalence of bovine tuberculosis in cattle began to rise inexplicably that there developed an appreciation of the significance of wild animal vectors. With the escalating prevalence in the early 1980s came the introduction of mass poisoning of possums (Tweddle and Livingstone, 1994).

The prevalence of bovine tuberculosis in New Zealand escalated in the early 1980s through to the early 1990s (Tweddle and Livingstone, 1994). At the end of 1994, the number of infected cattle herds was nearly three times that in 1980, the year when herd prevalence was lowest (Animal Health Board, 2000a). In areas where feral animal vectors were absent, the test and slaughter policy, as originally conceived, was, and still is, effective (Tweddle and Livingstone, 1994). The increase in herd prevalence seen through the 1980s was the result of the expanding problem of possum tuberculosis, a lack of resources for both feral animal vector control and for the control program in farmed livestock, plus the lack of knowledge of the epidemiology of tuberculosis where feral animal vectors were involved (Tweddle and Livingstone, 1994). Greater control and eventual eradication of tuberculosis from cattle and domestic deer is still the goal of the bovine tuberculosis program in New Zealand. The draft Pest Management Strategy proposed by the Animal Health Board (Animal Health Board, 2001) restated that intention.

The epidemiology of bovine tuberculosis in New Zealand is complex. *M. bovis* has been isolated from 14 different animal species (Morris and Pfeiffer, 1995; Table 1). However, the epidemiological picture was significantly clarified when the affected species were divided into those that were maintenance hosts and those that were spillover hosts. In a maintenance host infection persists without the need for the reintroduction of disease. That is, once infection is established a population remains continuously infected by intraspecies transmission. A maintenance host may be the source of infection for other species. In a spillover host, infection will not persist indefinitely unless there is re-infection from another species, although re-infection may be infrequent. Transmission to other species from a spillover host to domestic livestock may occur. That is, both maintenance and spillover hosts may act as disease vectors.

The established maintenance hosts for tuberculosis in New Zealand are the possum, cattle and deer (either domestic and feral) with most of the remaining species being spillover hosts. Most of the latter species are of little or no consequence in the maintenance

of infection or its transmission to domestic stock. The role of the ferret in the epidemiology of tuberculosis is not finally resolved. It cannot be considered a maintenance host over most of its range. However it may significantly amplify infection in some areas, for example, in North Canterbury (P. Caley, pers comm 2001).

In New Zealand the spillover hosts share habitat with possums and are infected from possums. Feral carnivores and omnivores become infected by scavenging the carcasses of dead tuberculous possums. Paradoxically, the prevalence of disease in some spillover species may be substantially higher than in the maintenance host, even where there is little or no intra-species transmission. In these species there is a high frequency of exposure and the disease takes a chronic course. Tuberculosis in feral carnivores and omnivores may be used to indicate the presence of tuberculous possums.

Table 1.1

Animal species found in New Zealand to be infected with *Mycobacterium bovis*

Species	Host status	Potential source of infection for domestic livestock
Brushtail possum	Maintenance	High
Deer - both domestic and feral	Maintenance	High
Cattle	Maintenance	High
Ferret	Spillover	Generally low to moderate but high in some specific areas
Pigs (feral)	Spillover	Low
Sheep	Spillover	Very low
Goat	Spillover	Very low
Rabbit	Spillover	Very low
Hare	Spillover	Very low
Hedgehog	Spillover	Very low
Stoat	Spillover	Very low
Weasel	Spillover	Very low
Cat	Spillover	Very low
Dog	Spillover	Very low

Ref: Morris and Pfeiffer, 1995

There is epidemiological evidence that deer played a central role in infecting possums with *M. bovis* (Morris et al., 1994). Deer develop open draining sinuses and possums may

have become infected from environmental contamination. Alternatively, possums may have eaten from a tuberculous deer carcass as they have been observed feeding on deer carcasses (Nugent et al., 2000). Ironically, the prevalence of tuberculosis in feral deer populations is exacerbated by tuberculosis in the local possum population (Nugent et al., 1997).

In cattle the principal route of transmission is respiratory with the majority of infected cattle having lung lesions (O'Reilly and Daborn, 1995). The predominance of aerosol transmission was established from the distribution of lesions in natural cases and in experimental infection studies (Francis, 1972; Lepper and Pearson, 1973). Infection passes from one animal to another in aerosols of droplet particles containing *M. bovis*. The infectious particles, termed droplet nuclei, form when the moisture evaporates from the aerosol droplets. Droplet nuclei, 3 - 10 μm in diameter, may contain up to 10 bacteria, and are aerodynamically very stable, remaining suspended in air for long periods (Smith and May, 1994).

Contamination of the physical environment with *M. bovis* is not a significant source of infection for animals. Considerable caution is necessary in inferring duration of infectivity in the environment from the studies of survival times in laboratory studies. Although large numbers of *M. bovis* bacteria are shed in faeces, pasture contamination is not an important source of infection, nor are fomites substantially involved. In humans ingestion is 10,000 times less effective than inhalation as a route of infection due to the killing of the bacteria by gastric secretions (Smith and Moss, 1994). Notwithstanding the difficulty of infecting animals by the oral administration of *M. bovis*, and the dilution in the environment of any excreted bacteria, the *M. bovis* does not survive long in the environment so there will be no build up of contamination. Some studies have demonstrated that *M. bovis* may survive for long periods in faeces, sewage and soil (Francis, 1947). The bacteria not only have to survive in the environment but in places where animals will be exposed to an infective dose. In two studies of *M. bovis* survival on pasture both reported short survival time (Duffield et al., 1985; Jackson et al., 1995a). In New Zealand the bacteria remained viable for less than 4 days on pasture and to a maximum of 4 weeks under the most favourable conditions (Jackson et al., 1995a). Environmental survival is affected adversely by ultra-violet light, high environmental temperature and low relative humidity (Duffield et al., 1985; Jackson et al., 1995a).

Though *M. bovis* may survive in possum carcasses for several months, the organism is not readily available to infect other possums or livestock.

Biology of the Brushtail Possum

General Description

Possums are solitary and strictly nocturnal, spending the daytime in dens (Cowan, 1990; Cowan and Clout, 2000). Individual possums use many different dens, up to 11-15 different dens in a year, but most dens are used only occasionally. They change dens frequently, spending on average two out of three days in different dens. Den sharing is uncommon, but dens often are used sequentially by different possums (Caley et al., 1999).

Most mature females produce one joey per year with the peak of matings occurring in March – April. Up to 40% of mature females breed again in the second mating period, September- October. The possum is a marsupial and the young, called a “joey”, is reared in a skin pouch on the female’s lower abdomen. The joey is born 18 days after conception as a very underdeveloped foetus, and spends 5 months in the mother’s pouch before leaving the pouch and becoming a semi-independent “backrider”. Juvenile possums, at approximately one year of age, become fully independent and establish their own territory. The majority of juvenile females remain within the home range of their mother but a small proportion of females and a larger proportion of juvenile males move away (disperse) from the area of their birth. Typically the dispersal distances are up to several kilometres and they may travel 20 kilometres or more (Cowan and Clout, 2000).

The home range characteristics of possums vary widely, and are dependent on the environment. The shape and size of home ranges are influenced by habitat, density relative to carrying capacity and absolute density although the relative influence of each feature is not known. Home ranges vary in size from about 1 to >30 hectares (Cowan and Clout, 2000). Possums confine their activity to their established home range and do not travel very far outside it. Brockie et al. (1989, 1997) found that possums could not be drawn out of their home range and would not travel more than approximately 300 metres to a bait station. Males generally have larger home ranges than females. Home ranges overlap extensively both within and between the sexes. Activity within the common areas of the home ranges is governed by mutual avoidance between co-dominant possums of each sex

(Winter, 1976, cited by Cowan and Clout, 2000). Possum populations are spatially clustered, and habitat and carrying capacity of different habitat types dictate the spatial distribution more than any tendency to form social aggregations (Efford, 2000).

Once established, an adult possum's home range is stable in both time and space, and they tend to occupy these home ranges for life. Removal of possums by control operations may result in some rearrangement of home ranges but only possums in the immediate area, those whose home range overlap the depopulated area, move to utilise the available resource (Efford, 2000). That is, possums show a limited tendency to actively spread themselves evenly over the available habitat. A shift of more than a few hundred metres is highly unusual for adults (Cowan, 1993). Not all of a possum's home range is used at any one time and seasonal movements to food sources constitute occasional use of specific parts at particular times. A possum may take 3 to 5 nights to completely cover its range (Cowan and Clout, 2000).

The "vacuum effect", the attraction of possums into a controlled areas from the areas immediately surrounding it, has been suggested as the reason for the rapid recolonisation of controlled areas and the apparent failure of buffer zones to control the spread of bovine tuberculosis. Efford et al. (2000) observed some local adjustments of home ranges by possums denning adjacent to the controlled area in the 2 months after a control operation. The effect was detectable for 200 – 300 m from the edge and involved only those possums whose existing home ranges overlapped the controlled area. There was no evidence of adult possums relocating over long distances. Numerous other studies in a variety of habitats and possum densities have failed to demonstrate a vacuum effect operating over more than few hundred metres (Green and Coleman, 1984; Cowan, 1993; Cowan and Rhodes, 1993).

Habitat Preference

Possum population densities vary greatly between major habitat types and even within broad vegetation types there is considerable variation. Densities decline with increasing altitude, a pattern driven more by altitudinal zonation of palatable vegetation than by physical variables of temperature and rainfall. The highest possum densities occur along a 250 m zone within a forest along pasture margins. Some of the possums within the zone feed on pasture plants, but Efford (1985, cited by Efford, 2000) found that females living

within approx. 200 m of the forest edge derived most of their food (about 93%) from within the forest. Males in the same zone derived twice as much of their food from the pasture, but that was still a small fraction of the total. The nutrition gained from pasture was an insufficient explanation to account for the population edge effect. The greater densities probably derive from greater diversity of food sources and greater biomass of the understorey along forest margins (Efford, 2000).

Population Structure

The equilibrium structure of an undisturbed possum population depends upon habitat type, food and denning resources (Efford, 2000). Without imposed controls populations range within $\pm 50\%$ of the long-term average carrying capacity. Females are longer lived than males with annual survival being 90% for those aged between 2 and 5 years, compared with 80% for males in the same age range. Recruitment almost exactly balances losses, with a high death rate in the young of each year. Even without the effects of imposed population control or natural causes of population change, the movement of juvenile possums contributes strongly to the structure and turnover of local populations. Dispersal from natal areas occurs at a constant rate, not affected by local densities or the densities in the surrounding areas (Efford, 2000).

Dispersal and Recolonisation

Juveniles become independent and establish their own home ranges usually before they are one year old (Cowan et al., 1997) with some doing so as late as 18 months (Efford, 2000). Possums that disperse long distances are almost always individuals that are undergoing or have recently undergone sexual maturation, and this group includes about four times as many males as females. Dispersal occurs most often about the time of the peak in breeding, late summer and early autumn or spring for those population that breed twice a year. The average distance traversed by a possum born in farm land or around a swamp in Hawkes Bay (where the movement exceeded 2 km) was in the range 3 - 11.5 km, and one possum moved 25 km (Cowan et al., 1996). Approximately 20% of juveniles undertake such movements. The dispersal may be done in a single move or over several consecutive moves. Female dispersal behaviour differs from that of males. Females tend to establish home ranges close to or overlapping that of their mother (Clout and Efford, 1984). If juvenile females disperse they move further and make more moves before settling (Efford, 2000). The biological force for dispersal is unclear. It is not dependent on density,

as juveniles move out from areas of low density, may traverse depopulated areas of good habitat and low density, and through areas with an excess of food and dens sites, all without settling.

One of the major reasons for the expansion of tuberculosis-affected areas is the dispersal of infected juvenile possums (Cowan et al., 1996). After natural or imposed population decline the population eventually recovers through a mixture of breeding by survivors and local recruitment of their offspring, plus immigration from surrounding areas. In the 2 – 3 years after an initial control operation, immigration is likely to be more important than breeding (Efford, 2000).

Social Structure

Possums are solitary when feeding and when moving around at night (Day et al., 2000). They are generally solitary when sleeping in dens with the clear exception of mature females with young less than a year of age. Mature males may also share with mature females during the breeding season. Possums may congregate around localised sources of abundant food but maintain some distance from each other when doing so (Day et al., 2000).

Possums do not defend a territory, but at low densities may maintain exclusivity in an area. Their social structure is founded on a dominance hierarchy and mutual avoidance (Clout, 1977, cited Day et al., 2000). They spend little time in direct social contact except during breeding. Den sharing is rare but they do not have exclusive use of dens. If challenged, both male and female possums will defend their dens. When feeding on a concentrated source of food, possum usually keep distant from each other and feed alone. They may defend high quality food sources, for example, bait stations, but will not continue to defend the site after they cease feeding (Day et al., 2000). However, the effects of population density, habitat, climate and demographic structure on social organisation and behaviour are not fully understood.

Tuberculosis in Possums

The first report of naturally acquired bovine tuberculosis in a feral possum was in 1967 (Ekdahl et al., 1970). It was found on a farm on the West Coast of the South Island where

tuberculosis was a persistent problem in cattle. It is not known for how long tuberculosis had been present in possums prior to its first recognition in 1967. Circumstantial evidence would suggest that infection had been established a decade or more earlier and had spread relatively slowly in the local population (Morris and Pfeiffer, 1995). However, soon after the first report, further cases were found in a number of widely scattered locations involving both the North and the South Islands. To account for this pattern of disease, Morris and Pfeiffer (1995) postulated that infection probably entered the possum population during the decades of the 1950s, 1960s and 1970s with feral deer the principal source of infection. Transmission from deer to possums in as few as 50 locations during that period would account for the pattern of disease reported (Morris and Pfeiffer, 1995).

Pathology and Transmission

Tuberculosis in possums is usually progressive and fatal. In possums it is principally a respiratory infection, with 85% of tuberculous possums having lung lesions (Jackson et al., 1995b). The diagnosis of tuberculosis can only be made with high sensitivity by post mortem examination. Though clinical signs are a valuable indicator, they are less than 75% sensitive but highly specific (Jackson et al., 1995c). From the site of primary infection, the disease spreads rapidly to other organs, and, body and superficial lymph nodes. Affected lymph nodes become enlarged and contain caseous, pale yellow semi-fluid pus. The swollen lymph nodes may rupture through the skin to form draining sinuses. The clinical signs of infection include the presence of palpable swellings in peripheral body lymph nodes of the axillary and inguinal regions, the lymph nodes of head, and debility and wasting. In possums with superficial lymph node lesions, 45% were found to have formed draining sinuses (Cooke et al., 1995). Lesions contain large numbers of *M. bovis* organisms. Terminally-ill tuberculous possums have extensive lung lesions and excrete large numbers of *M. bovis* in tracheal exudates. In a study of 73 tuberculous possums macroscopic lesions were found most frequently in the lungs (75%). The number of sites with lesions ranged from 1 - 10 (mean 4.6), and a wide range of anatomical sites were involved (Jackson et al., 1995c). Microscopic lung lesions were found in 85% of infected possums, with a range of 1 - 28 sites and the mean number of sites 11.6. This pattern of infection indicates early generalisation of infection.

There are two principal transmission pathways for possum to possum transmission of tuberculosis: pseudovertical between mother and joey and direct horizontal transmission

via infectious aerosols between adults (Pfeiffer, 1994). Pseudovertical transmission between mothers and their joeys occurs during the long period of pouch life up until independence. It is the method of spread from one generation to the next. The potential transmission pathways include aerosol from respiratory lesions, the oral route of transmission from infected mammary glands and possibly direct cutaneous infection from contaminated saliva. Direct horizontal transmission is the method of spread within generations. The respiratory route is the principal route of transmission between adult possums (Jackson et al., 1995b). It has been postulated that transmission occurs through intimate social interactions, when competing for dens, during simultaneous den sharing, during courtship and mating, and between males during competition for females. Studies on environmental survival have relegated indirect transmission to a very minor role, both in the transmission from possum to possum and between possums and livestock (Jackson, 1995).

Bovine tuberculosis kills possums but at prevalences found in most cross-sectional studies, between 1 - 10%, it does not appear to affect local population size (Coleman and Caley, 2000). The length of time from infection to expression of clinical disease is unknown and it is believed that the progression from pre-clinical to clinical disease is a function of the combined effects of severe environmental changes and other stresses, including the stress of reproduction (Pfeiffer, 1994). Once clinical signs are detected 50% of possums died within 2 months (Pfeiffer, 1994), but one clinically tuberculous possum was known to survive for 15 months (Jackson, 1995).

The evidence for possums being a significant maintenance host is very strong (Morris and Pfeiffer, 1995):

1. there are spatial and temporal associations between tuberculosis in possum populations and the incidence of tuberculosis in domestic stock,
2. infection is endemic in possum populations without the need for continued reinfection from other wild animals or domestic stock,
3. when tuberculous possum populations are reduced, and therefore the number of tuberculous possums also, there is a long term decrease in the incidence of infection in domestic cattle and deer, and,
4. restriction endonuclease analysis (REA, a means of subspeciating *M. bovis*) of isolates of *M. bovis* from longitudinal studies show the same subtypes persist in possum

populations for extended periods and is consistent with long-term maintenance of infection.

Spatial Distribution of Tuberculous Possums (Hotspots)

Tuberculosis in possum populations is spatially and temporally aggregated, in what have been colloquially termed "hotspots". Spatially, tuberculous possums are many times more likely to be found denning, or to be trapped, within 50m of another tuberculous possum. Some of these hotspots have been shown to persist for up to 25 years (Coleman and Caley, 2000) and in spite of population control programs (Caley et al., 1999).

Pfeiffer (1994) hypothesised that the spatial component of the persistence of hotspots was due to the combined effect of pseudovertical transmission, daughters establishing a home range that overlaps with that of their mother, aggregated mating patterns and environmental stresses. Temporal clustering was postulated as resulting from environmental stresses, including low temperatures, high rainfall and poor nutrition.

Transmission of Tuberculosis from Possums to Livestock

It was the frequency with which draining sinuses were observed in infected possums that led to the initial theory that the main route of transmission between possum and livestock was by way of pasture contamination (Julian, 1981). It was postulated that tuberculous possums spread pus onto pasture and cattle were infected by ingestion of contaminated pasture. However, there is a low probability of livestock becoming infected by ingesting pasture contaminated with *M. bovis*, as very high doses, in the order of 10^7 – 10^9 bacteria are required to initiate infection by this route (Frances, 1947).

Observations on cattle and deer behaviour showed that transmission of infection probable occurs by inhalation of an infectious aerosol. Possums in the terminal stages of disease have been observed and their behaviour is demonstrably different to that of healthy possums. Normally nocturnal, agile and wary animals, when terminally ill they became debilitated, were active during the day, were less inclined to retreat to the safety of dens; they appeared dazed, and developed a staggering gait. They were unable to avoid the approach of inquisitive livestock attracted by the abnormal behaviour (Paterson and Morris, 1995; Sauter and Morris, 1995). In simulation studies using sedated possums,

cattle and deer investigated the possum and were seen to lick, bite and sniff the possum. During such investigations of a terminally ill possum there would be ample opportunity for transmission infection by aerosol.

Control of Possum Tuberculosis

As of June 2000, tuberculous possums occupied 8.95 million hectares, or approximately 33% of the land area of New Zealand (AHB 2001). Diseased populations were present in 28 discrete areas, two main areas in the North Island (the central region and the Wairarapa) and three main areas in the South Island (Westland, North Canterbury and Otago; Coleman and Livingstone, 2000). These areas also contained approximately 75% of the infected cattle and deer herds in the country.

The control of tuberculosis in possums is a complex process and involves an understanding of the mechanisms of transmission of *M. bovis* between possums, and from possums to cattle and deer, as well as the biology and behaviour of possums and population dynamics. The current method of controlling tuberculous possum populations is culling. The population in areas where tuberculosis in possums is endemic are reduced by poisoning and trapping. Culling is effective in reducing the incidence of tuberculosis in livestock, with the effect being seen in the years following the control (Tweddle and Livingstone, 1994; Pannett, 1995; Caley et al., 1999).

In a control program, the initial cull of an area aims to reduce the possum population to 10% of the pre-control level. Follow-up culls, conducted annually or biennially, are undertaken to maintain the population at or below the 10% level (Coleman and Livingstone, 2000). Although population control can suppress the transmission of tuberculosis to livestock for an extended period, such control measures do not lead to disease eradication in the possum populations. Without ongoing maintenance control of the possum population, tuberculosis levels in livestock usually returns to pre-control levels in 8 to 10 years as possum numbers increase (Barlow, 1991). The rate and scale of movements by possums in recolonising controlled areas, along with reproduction and survival rates, dictate the frequency with which control must be carried out to keep disease transmission risks at acceptable levels (Efford, 2000).

Control of infected populations by culling is costly, requiring regular maintenance control and it does not eradicate tuberculosis. In most areas population control is undertaken for two reasons. Firstly to decrease the spread of tuberculosis to domestic livestock by reducing the population of diseased possums, and secondly to restrict the spread of tuberculous possums into adjacent populations that are free of disease. Where possible, natural barriers to possum movement, such as mountain ranges and rivers, are employed to help prevent the movement of diseased possums (Tweddle and Livingstone, 1994).

Managing possum dispersal is the most important requirement in limiting the extension of possum populations infected with bovine tuberculosis and the recovery or repopulation of areas after control. When a population is culled in the area surrounding a known focus of tuberculous possums, the area is referred to as a "buffer zone". Buffer zones are areas where possum density relative to carrying capacity is kept very low. The concept underlying the function of a buffer zone is based on known dispersal behaviour of juvenile possums, and the width of the zone is determined from knowledge of dispersal patterns. The underlying assumption is that dispersion of possums is controlled by population density and the force for dispersion can be neutralised by the "vacuum effect". The long distances moved by juvenile possums and their tendency to disperse even when population densities are low means that buffer zones used for control of possum population will always be 'leaky'.

It was initially contended that eradication of tuberculosis from a possum population was possible by merely maintaining the population at a level below 50% of its carrying capacity for 10 years (Roberts, 1996). This was found to be simplistic and misguided as epidemiological research showed that tuberculous possums are not found evenly distributed throughout contiguous possum populations but are clustered in both time and space. The clustering makes the control of possum tuberculosis using broadscale population reduction measures very difficult. It has been proposed that vaccination may be an alternative control strategy.

There are three sources of *M. bovis* that may lead to infection of the re-emerging population: survival of *M. bovis* in the environment, immigration of infected possums, and

infected possums surviving an incomplete culling program. The relative importance of these three sources has not been determined.

BCG Vaccine in Humans and Domestic Livestock

Bacille Calmette-Guérin (BCG) vaccine is a live attenuated strain of *M. bovis*. It has been used for the prevention of tuberculosis in humans since 1921 (Bloom and Fine, 1994). BCG vaccine has an exemplary safety record (Hanson et al., 1995; WHO, 1995b). Overall, approximately 3 billion doses have been administered, and of these 2 billion doses were given to newborn infants (WHO, 1995b). BCG is the most widely used human vaccine in the world, being used in 172 countries (WHO, 1995b). It is part of the childhood immunisation program in many countries and is often administered at birth (Guerin et al., 1999).

The efficacy of BCG in humans has been in contention for over 50 years. Between 1927 and 1968, 21 control trials of BCG were undertaken and the efficacy ranged from 0 to 80%. Between 1988 and 1995, 14 case-control studies were done and vaccine efficacy ranged from 2% to 83%. However, there is no consensus on the meaning of the results of these trials because there were many differences between them, both in design and in the vaccine used. Different strains of BCG were used, the vaccines were manufactured and stored in different countries and different regions (WHO, 1995b). A meta-analysis of 10 randomised control studies and eight case-control studies was done by Rodrigues et al. (1993). When analysed in this way there was a consistently high protective effect (75% - 86%) against meningitis and millitary tuberculosis, the form of disease mainly found in children. No consistent protective effect against pulmonary disease was found.

In humans vaccination does not appear to prevent primary infection with *M. tuberculosis* nor does it prevent an appreciable number of infectious pulmonary cases (Colditz et al., 1995). Therefore, it would not significantly decrease transmission of tuberculosis in a community. BCG has its greatest value in protecting children (WHO, 1995b).

BCG vaccination in most recipients causes conversion to tuberculin skin test positivity (Bloom and Fine, 1994). The duration of the hypersensitivity is variable and the level of

reactivity wanes with time. There is no definitive evidence that BCG re-vaccination confers additional protection against tuberculosis in humans, irrespective of their tuberculin status before or after vaccination (Karonga Prevention Trial Group, 1996; Reider, 1996) and WHO discourages BCG revaccination (WHO, 1995b). There is minimal risk in administering BCG vaccine to people with a positive tuberculin reaction, even if the reaction is due to natural infection or prior BCG vaccination (WHO, 1995b). An argument against adult BCG vaccination is that the resulting tuberculin hypersensitivity makes the interpretation of the tuberculin skin test difficult (Harries et al., 1997).

As with its use in humans, BCG vaccination of cattle has been dogged by controversy (Newell and Hewinson, 1995). The reported levels of vaccine efficacy ranged widely. As with the human vaccine trials with BCG, there were differences in the vaccine, the strains of BCG used, and the design of the experiments, all of which render direct comparisons of results difficult. Recent studies in cattle using low doses of BCG however have demonstrated high levels of protection (Buddle et al., 1995a). Previous studies may have failed, among other reasons, because they used high doses of vaccine, in the order of 10^8 to 10^{10} cfu (Griffin et al., 1999).

Vaccination of cattle, although capable of protecting against tuberculosis, is unlikely to be used in New Zealand as it may induce tuberculin reactivity. As the skin test is the basis of the national tuberculosis control program, it would no longer be useful in vaccinated cattle. The economic cost to farmers from tuberculosis relates to the restrictions on the movement of their cattle. Therefore control strategies must to be aimed at eliminating disease so enabling farms to trade freely. Vaccination would not be cost effective as it is unlikely to be 100% effective under conditions of challenge from tuberculous possums. In that circumstance farmers would be in the worst of all situations, with the disease present in their livestock but without the ability to differentiate infected from vaccinated animals. In addition, international markets may impose trade barriers on meat from vaccinated cattle or subject it to restrictions.

Vaccination of deer by subcutaneous inoculation was effective in protecting deer against establishment of disease following experimental challenge (Thomson et al., 1995). Griffin et al. (1999) reported that when deer were given two doses of vaccine, it protected them against disease and infection. Outbreaks of tuberculosis in domestic deer may be very

dramatic with rapid spread of infection. Vaccination of deer could be used to control these explosive outbreaks where testing alone has been inadequate.

BCG Vaccination of Possums

The existing strategies for the control of tuberculosis in possums have been effective in reducing possum numbers and thereby the number of tuberculous possum in an area. However where possum control has been used, there is rapid recolonisation when the control program ceases. These control programs rely heavily on the use of toxins and there is growing public concern over the continued use of large quantities of poisons. Culling programs are costly and alternative, more cost-effective programs are needed. A promising option is vaccination of wildlife and a second, long term option, is biological control (Buddle et al., 2000).

The goal of wildlife vaccination would be to control infection in the animal reservoir and possibly the eventual eradication of infection. The initial target would be to reduce the susceptibility of the population to infections, and reduce the excretion of *M. bovis* from infected animals. These outcomes would break the chain of infection between possums and from possum to domestic livestock. To achieve these goals a vaccine would not, of necessity, have to prevent infection, but rather reduce the prevalence and incidence of infection to below a level capable of sustaining the disease in the population.

Aldwell et al. (1995a, 1995b) reported that BCG vaccination of possums could reduce the severity of disease and the degree of dissemination of infection after experimental challenge. Subcutaneous, intratracheal, and intranasal aerosol administration of BCG all proved effective. Buddle et al. (1997) reported that BCG when delivered directly into the duodenum provided a level of immunity equivalent to subcutaneous, intratracheal and intranasal vaccination. Neither intragastric nor oral administration was effective, presumably because the bacteria were destroyed by gastric secretions (Aldwell et al., 1995a, 1995b; Buddle et al., 1997). For oral delivery some form of encapsulation would be required to overcome the vulnerability of the bacteria to gastric secretions. Should that be achieved delivery of BCG as an oral bait could be used in a similar fashion to toxin baits.

Based on the above, two methods of delivering BCG vaccine to wild possums, aerosol and in baits, are feasible, at least in principle. Aerosolised vaccine could be delivered from a device designed to spray a possum when it enters, or BCG could also be incorporated in bait but protected from gastric secretions, and the baits could be used akin to toxin baits. If the delivery was safe and cheap, farmers could incorporate vaccination as part of their on-farm control program. Vaccination could also be used on a larger scale as part of an integrated regional area control strategy.

Outline of the research program in this thesis

The objective of my research program was to gain greater understanding of the potential of BCG as a vaccine to protect possums against tuberculosis. From this program of study we planned to develop a strategy for the utilisation of BCG vaccination, or any vaccine developed in the future, for use in wild possum populations. When the project commenced there had been substantial investment in New Zealand into tuberculosis vaccine development. The benefits of that effort would be gained only if the vaccine was effective and could be delivered to wild possums. Before vaccination could be adopted as a strategy for controlling tuberculosis, a number of questions needed to be addressed. In order to determine the practicability of vaccinating wild possums, and in the context of a field disease control program, a series of research projects were undertaken. The research that makes up this thesis was a series of studies aimed at addressing some of these outstanding questions and advancing the use of vaccination as a tool for controlling tuberculosis in wild possums.

NB. Details of the references cited in this section are listed at the end of the thesis.



Photographer: Sam Beckett

SECTION B.

BCG Vaccination Studies Using Captive Possums

Introduction

The objective of the research program was to gain a greater understanding of the potential of BCG as a tuberculosis vaccine for possums. This was to be achieved through a series of studies with captive possums and the examination of the effect of vaccination on tuberculosis in a wild possum population. The program and results are described in the following eight chapters.

Buddle and colleagues had conducted vaccination and challenge studies with BCG vaccine and individually caged possums. They had shown that BCG vaccine could be effective when administered by a number of different routes including intranasal aerosol. Intranasal aerosol vaccination was chosen for the initial studies in the present series. It was an easy procedure to perform and could potentially be used for vaccinating wild possums using a possum-activated vaccination-bait station. BCG vaccine was used as it had been effective in the initial cage studies and no other vaccine was available.

The initial studies demonstrating BCG vaccine efficacy were conducted at Wallaceville. When possums are caged they exhibit signs of stress (Presidente and Correa 1981, Buddle et al., 1992) and stress depresses immune responses. However, even under these conditions vaccinated possums still performed better than controls in experimental challenge studies. In housing possums in large outdoor enclosures in a bushland setting, we hoped to decrease the stresses associated with confinement and so be able to better assess the levels of protection afforded by BCG vaccination, minimally confounded by intercurrent stress.

Before vaccination could be considered for field use and before field trials could be undertaken, a number of questions had to be addressed. Questions on the duration of the protective response and the effect of revaccination were addressed in the first two studies. The need for the third experiment arose out of considerations on the design of a possum-activated vaccinator-bait station.

The first experiment (Chapter 2) had several objectives. The first was simply to determine the level of protection induced when a single dose of BCG was administered as an intranasal aerosol. The second objective was to address the question of the longevity of

protection induced by a single dose. For use in the field it was necessary to know how long protection from a single dose would last - was it lifelong or would periodic boosting be necessary?

The second experiment (Chapter 3) was designed to investigate the effect on protection of repeated doses of vaccine. Under field conditions the frequency of re-vaccination will be beyond immediate control and some possums may be exposed repeatedly over a short period of time or revaccinated after a period of months. It was not possible to predict the effect on the possum's immune system of these repeated exposures. In immunology circles opinion was divided on the effect on protective immunity of multiple exposures to antigens that stimulate a cell mediated immune (CMI) response. In a broader context, the number of times any wild host may be exposed to vaccine will largely be beyond control and so the answer to this question would have implications for the use of all vaccines for wildlife, for all modes of delivery and all wild life species where the vaccine relies on stimulating CMI for protection.

The third experiment (Chapter 4) addressed vaccination by instilling BCG vaccine onto the conjunctiva as an additional route of vaccination. The need for this experiment arose when we were considering the design of a device to deliver an aerosol vaccine to wild possums, in the form of a specialised vaccinator-bait station. Possums will investigate novel objects in their environment, and by utilising this aspect of a possum's nature, a possum-activated vaccinator that would spray vaccine into a possum's face, could be designed. Greater flexibility in the design of the vaccinator, and a greater probability of delivering an immunising dose would be achieved if the vaccine were sprayed onto the possum's face, into the eyes as well as into the external nares. However, nothing was known about the clinical and immunological effect of administering BCG vaccine to the conjunctiva.

Chapter 2

Aerosol vaccination of the brushtail possum (*Trichosurus vulpecula*) with bacille Calmette-Guérin: the duration of protection *

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ABSTRACT:

Bovine tuberculosis is endemic in wild brushtail possums (*Trichosurus vulpecula*) in New Zealand. The disease is controlled by reducing or eliminating infected possum populations, but control methods do not kill all possums in the targeted area, leaving some tuberculous possums to maintain the disease. Vaccination with bacillus Calmette-Guerin (BCG) has been shown to provide significant levels of protection. Vaccination is a potential alternative or complementary control strategy if protection is long lasting. Captive possums were vaccinated with a single dose of BCG by intranasal aerosol and challenged by intratracheal instillation of *Mycobacterium bovis* two, six or 12 months after vaccination. Vaccination produced significant immunity as measured by the lymphocyte proliferative response to bovine PPD and protection in response to challenge. The protective response was seen as a decrease in the mass of pulmonary lesions and decreased dissemination to the abdominal organs and body lymph nodes. The protective effect was strongest at 2 months after vaccination but was still present at a lower level at 12 months. Delivery of an aerosol vaccine to possums in the wild using a self-delivery system could contribute substantially to wildlife tuberculosis control.

INTRODUCTION

Bovine tuberculosis in wild animals, which act as reservoirs of infection for domestic animals, other wild animal species and humans, is becoming a world wide problem. Significant wild animal reservoirs have been identified in cervids in North American (Schmitt et al., 1997), in badgers in the United Kingdom and Ireland (O'Reilly and Daborn, 1995; Hughes et al., 1996), in Cape buffalo in South Africa (O'Reilly and Daborn, 1995), and brushtail possum in New Zealand (Morris et al., 1994). Vaccination of wild animals may be the only alternative to culling as a means of controlling infection in wild populations. Vaccination would be the preferred option for wild animal species of high economic or high conservation value (Hughes et al., 1996).

In New Zealand, the introduced Australian brushtail possum (*Trichosurus vulpecula*) is the major wildlife reservoir of *Mycobacterium bovis* (Aldwell et al., 1995a) for domestic cattle and deer, and also a significant environmental pest. Infection

is endemic in possum populations in many areas of both North and South Islands. Infected possum populations are culled by trapping and poisoning programs to reduce the risk of infection in domestic stock (Caley et al., 1999). Control of possum tuberculosis by population reduction is a costly and unending process, for, without continued control of the population, numbers rapidly recover and with it tuberculosis prevalence. In the long term, new strategies for control of tuberculosis in possum populations will be required. Vaccination of possums against tuberculosis is a promising option (Buddle et al., 2000).

Vaccination with bacillus Calmette-Guerin (BCG) has been shown to give protection against experimental challenge with *M.bovis* in cattle (Buddle et al., 1995), deer (Griffin et al., 1999) and brushtail possums (Aldwell et al., 1995a, 1995b; Buddle et al., 1997). Studies in humans from several parts of the world have reported the protective efficacy of BCG as ranging from negative to more than 90% (Rodrigues et al., 1993). Possums when challenged seven to nine weeks after vaccination with BCG by various routes showed protection, and vaccination significantly decreased the severity of disease and the dissemination of infection to the liver and spleen (Aldwell et al., 1995a, 1995b; Buddle et al., 1997). BCG vaccination by intranasal aerosol (Aldwell et al., 1995b), subcutaneous inoculation (Aldwell et al., 1995a, 1995b), intratracheal (Aldwell et al., 1995b) and intraduodenal (Buddle et al., 1997) instillation have all been shown to be efficacious, but oral administration was ineffective (Aldwell et al., 1995a). The latter authors concluded that given a suitable delivery system, aerosol vaccination of possums, used in conjunction with other control measures, might be a suitable method for reducing the spread of *M.bovis* from wildlife to domestic animals (Aldwell et al., 1995a). Mathematical modelling of tuberculosis in wild possum populations predicts that prevalence could be reduced, possibly the disease eradicated using a vaccine, even where the vaccine had less than 100% efficacy and only half the possum population was effectively protected (Roberts 1996).

A protective effect of BCG in possums has been demonstrated seven to nine weeks after vaccination (Aldwell et al., 1995 a, 1995b; Buddle et al., 1997) but, for field application, protection over a longer period would be essential. The aim of our study was therefore to examine the duration of the protective effect of a single dose of BCG

vaccination administered as an intranasal aerosol. In these studies we examined the level of protection at two, six and 12 months after vaccination.

MATERIALS AND METHODS

Animals

Eighty-three adult male possums weighing 1.5 kg to 3.8 kg were trapped in a rural area of the North Island (NZ), an area free of possum tuberculosis. The possums were housed communally outdoors, in large wire mesh-covered pens and acclimatised to captivity for a minimum of 42 days prior to experimentation. Vaccinates and controls were held in separate pens for two weeks after vaccination to minimise the risk of BCG spreading to the controls.

For weighing, bleeding and vaccinating, the possums were sedated with 100 to 150 mg of ketamine hydrochloride (Parnell Laboratories, East Tamaki, NZ) (Buddle et al, 1994). The possums were examined visually each day, and weighed every 3 to 4 weeks. Before administration of the challenge inoculum, possums were sedated with 70 mg of ketamine, and then anaesthetised with 1ml of Saffan (12 mg/ml; Pet Elite Ltd, Lower Hutt, New Zealand) administered by intramuscular injection. Possums were euthanized by the intraperitoneal administration of an overdose of pentobarbitone while under ketamine sedation.

Experimental design

Possum were vaccinated on the same date and challenged at two, six and 12 months after vaccination. At each time point, nonvaccinated possums, trapped at the same time and held for the same time, were similarly challenged. We repeated the two-month post vaccination challenge trial (two-month Trial B) with possums captured in February 1997. Possums trapped in October 1996 were allocated to one of three groups, to be challenged at two months (20 possums), six months (20 possums) or 12 month (23 possums). Approximately half of each group was vaccinated (Table 2.1). Allocation to vaccinated or nonvaccinated control subgroups was done systematically from a list of all possums ranked on body weight. Possums for the two-month Trial B were allocated to treatment groups alternately from a list ranked on body weight. At the time of

vaccination the median weights of all subgroups were similar. Vaccination of the first three groups was done in December 1996 and of two-month Trial B in April 1997.

Vaccination

BCG Pasteur strain 1173P2 was prepared as described by Aldwell et al (1995b). Briefly, broth cultures were grown to mid-log phase in Tween albumin broth (TAB), the number of organisms was estimated from the turbidity of the culture and dilutions were made in sterile TAB. The number of colony forming units (cfu) was determined, after the vaccine suspension had been used, by plating 10-fold dilutions on supplemented Middlebrook 7H11 agar.

Possoms were vaccinated once only. The possums were sedated and the vaccine was administered as a metered aerosol spray (Valois Spray atomisers, Douglas Pharmaceuticals Limited, Auckland, NZ) of 100 µl directed at each nostril from a distance of 1 to 2 cm. The BCG vaccine suspension used in December 1996 and that used in April 1997, contained 6.5×10^6 cfu and 5×10^6 cfu per ml, respectively.

Challenge

The challenge suspension was prepared from *M.bovis* strain 83/6235, originally isolated from a naturally-infected possum (Buddle et al., 1994). The challenge inoculum was prepared in the same way as the BCG suspension. Possums in the vaccinated and control groups were challenged in the same way. A 1.5 mm external diameter plastic cannula was passed through the mouth and down the trachea until the end of the cannula lay beyond the level of the tracheal bifurcation. Once in place, 200 µl of a *M.bovis* suspension was instilled into the lungs and the cannula was flushed with an equal volume of sterile saline. After infection the possums were placed in left lateral recumbency to recover. Possums in 2-month Trial A were challenged with approximately 28 cfu, those in 2-month Trial B and the 6-month trial with 78 cfu and those in the 12-month trial with 50 cfu.

Necropsy

All possums were subjected to a detailed post mortem examination. All possums surviving to the end of the 7th week after challenge, were euthanased. *Post mortem*

body and lung weights were recorded. At the necropsy examination, the distribution of macroscopic lesions was recorded. A sample of lung (approximately 2 g) from a typical tuberculous abscess and one-third of the spleen was collected from each animal for bacteriological examination.

Tissue for histological examination was preserved in 10% buffered formalin. The following lymph nodes were collected: mandibular, parotid, deep cervical, caudal cervical, deep axillary, superficial axillary, inguinal, iliac, hepatic, gastric, mesenteric, and mediastinal. Other tissues collected for examination were the tonsils, liver, lung, spleen, kidneys and adrenal glands. For examination, tissues were embedded in paraffin, sectioned at 3 μm , stained with haematoxylin and eosin and by the Ziehl-Neelsen method. Histological examination consisted of detection of tuberculous granulomas, characterised by the presence of acid-fast bacteria within the lesions. A tissue was classed as positive if it contained one or more tuberculous granulomas

Bacteriology

Bacteriological examination consisted of decontamination of the macerated specimen with 0.75% cetylpyridinium chloride for 1 hour and centrifugation at 3,500 *g* for 20 min. The deposit was resuspended in sterile distilled water and inoculated onto Lowenstein Jensen agar supplemented with pyruvate and modified Mycobacteria 7H11 agar containing 10% sheep serum and 0.5% lysed sheep red cells. Inoculated media were incubated at 37°C and inspected weekly. The identification of the mycobacteria isolated was determined as previously described (de Lisle and Havill, 1985).

The concentration of *M. bovis* in lung and spleen was determined by the appropriate dilution of homogenised tissue in TAB. Plates of modified 7H11 agar were inoculated with 0.1 ml of a 1/10 and 1/1000 dilution of the homogenised tissue.

Lymphocyte proliferation assay (LPA)

Whole blood preserved in heparin was collected for the lymphocyte proliferation assay (LPA) before and four weeks after vaccination, and in two-month Trials A and B blood was also collected at eight weeks after vaccination. Proliferation responses of peripheral blood lymphocytes were measured using the method described by Buddle et al (1994). Briefly, 1 ml of blood was added to 50 ml of lysing buffer containing 0.17 M

Tris and 0.16 M NH_4Cl , pH 7.2 and incubated at 37°C for 10 min. The cell suspension was centrifuged at 350 g for 10 min, resuspended and washed twice in PBS. The cells were finally resuspended in RPMI 1640, containing 2% normal possum serum, 2 mM glutamine and antibiotics. To each well of a sterile cell culture microtitre plate was added 200 μl of cell suspension containing approximately 1×10^6 mononuclear cells/ml. Cells were cultured with bovine purified protein derivative (PPD; CSL Ltd, Melbourne, Australia), 60 $\mu\text{g/ml}$ final concentration, or Concanavalin A (Sigma, St Louis, MO, USA), 5 $\mu\text{g/ml}$ final concentration. For the unstimulated control wells, 50 μl of RPMI 1640 medium was added. Assay conditions, harvesting and β -scintillation counting were as described previously (Buddle et al., 1994). The proliferative response to bovine PPD was expressed as the difference in mean count before and 4 weeks after vaccination.

Statistical analysis

The following parameters for the vaccinated and non-vaccinated subgroups at each challenge were compared using the nonparametric Mann-Whitney test: body weight at challenge, the proportional change in body weight after challenge, lung weights as a proportion of the post mortem body weight, bacterial counts from the lung, and bacterial counts from the spleen. Lung weight was taken as an estimate of the mass of lung lesions. For analysis, bacterial counts were \log_{10} transformed.

In the lymphocyte proliferation assay, the response to bovine PPD before vaccination was compared with that four weeks after vaccination. In order to achieve normality, the raw counts per minute (cpm) were \log_{10} transformed and the differences compared using the unpaired t test. Comparisons were made between vaccinates and controls in each trial.

Table 2.1.

Response of possums, vaccination with bacillus Calmette-Guerin by intranasal aerosol, to challenge with *Mycobacterium bovis* two, six or 12 months after vaccination.

Trial	Treatment subgroup	No. in subgroup	Challenge wt (kg)	Weight change / challenge wt (kg / kg) ^a	Lung wt / post mortem wt (g / kg)	Lung bacterial count (log ₁₀ cfu / g)	Spleen bacterial count (log ₁₀ cfu / g)	No. of Tissues with microscopic lesion ^b
2-month Trial A ^c	Control	9	2.5 (2.0, 2.8) ^d	-0.25 (-0.35, +0.08)	40 (18, 51)***	7.0 (7.0, 7.4)	3.8 (2.0, 4.8)**	28/103 (27.2%)***
	Vaccinates	11	2.6 (2.0, 3.1)	-0.20 (-0.34, +0.20)	27 (9.2, 36.5)***	7.0 (4.4, 7.2)	2.3 (0.3, 5.0)**	4/135 (3.0%)***
2-month Trial B	Control	10	2.6 (2.4, 3.3)	-0.25 (-0.35, -0.07)	26 (18, 45)	6.8 (5.5, 7.6)	2.2 (0.3, 3.3)	6/97 (6.2%)
	Vaccinates	10	2.6 (2.5, 3.1)	-0.19 (-0.42, -0.13)	27 (16, 51)	5.8 (4.4, 7.1)	0.3 (0.3, 3.3)	9/114 (7.9%)
6-month	Control	10	2.7 (2.5, 3.1)	-0.31 (-0.43, -0.19)**	32 (24, 49)	7.1 (6.1, 7.5)	4.1 (2.9, 5.4)***	39/130 (30.0%)***
	Vaccinates	10	2.7 (2.5, 3.1)	-0.21 (-0.36, -0.15)**	28 (18, 43)	6.8 (6.5, 7.7)	2.4 (1.7, 3.4)***	14/127 (11.0%)***
12-month	Control	10	2.9 (2.3, 3.3)	-0.29 (-0.35, -0.16)	25 (19, 42)	6.8 (4.0, 7.3)	2.6 (1.3, 5.4)	28/167 (16.8%)***
	Vaccinates	13	3.1 (2.5, 3.5)	-0.31 (-0.40, -0.10)	28 (19, 39)	6.9 (4.0, 7.5)	2.2 (0.3, 3.3)	11/161 (6.8%)***

^a Weight changes after challenge as a proportion of weight at challenge

^b The number of tissues, excluding those of the thoracic and abdominal cavities, with microscopic lesions divided by the number of tissues examined

^c The Mann-Whitney U test was used to test for statistical significance except for the comparison of number of lesions where the Chi-squared test was used. Asterisks indicated the level of statistical significance, * p<0.10, ** p<0.05 and *** p<0.01.

^d Median with minimum and maximum values in parentheses

Tissues examined histologically were grouped into three anatomical regions: the thoracic cavity (lung, tracheobronchial lymph nodes), abdominal organs (spleen, liver, hepatic and mesenteric lymph nodes, adrenal glands and kidneys), and body lymph nodes (mandibular, parotid, deep cervical, caudal cervical, superficial axillary, deep axillary, inguinal, and iliac lymph nodes, and the tonsils). The numbers of histological lesions in each region in vaccinates and controls in each trial were compared using the Chi-squared test.

Statistical analyses were conducted using SPSS for Windows version 9.0 (SPSS Inc., 1999).

RESULTS

Lymphocyte Proliferation Assay

The difference in the response to bovine PPD between the control and the vaccinated subgroups, measured at four weeks after vaccination, is shown in Table 2.2. There were significant differences between the vaccinates and the controls in two-month Trial A, six-month Trial, and 12-month Trial but not in two-month Trial B. In two-month Trials A and B, (the only groups examined eight weeks after vaccination) the response to bovine PPB peaked at four weeks and had declined by eight weeks after vaccination (data not shown).

Clinical findings after challenge

Clinical signs were seen at five weeks and onwards in those possums with advanced tuberculosis. Wasting was seen in most possums, diarrhoea occasionally and, in one instance, a possum showed laboured breathing. Possums that succumbed to infection were usually found dead on the ground or in a den. For each challenged group, there was a conspicuous decrease in food intake during the period from six to eight weeks after challenge. No palpable lesions were detected in any possum when they were examined four weeks after challenge or during *post mortem* examination.

At the time of challenge, the median weight of the subgroups in the two-month Trials A and B, and six-month groups were similar. For the 12-month challenge groups, the median weight of vaccinates (3.08kg) was greater than for the controls (2.84kg) but the difference was not significant.

Table 2.2

Lymphocyte proliferation response of possums to intra-nasal aerosol vaccination with BCG: the change in the response to bovine PPD four weeks after vaccination compared to non-vaccinated controls.

Trial	Treatment Subgroup			
	Control		Vaccinates	
	Mean	sd	Mean	sd
2 month Trial A ^a	0.234 **	0.255	0.794 **	0.248
2 month Trial B	0.151	0.448	0.219	0.697
6 month	0.208 *	0.241	0.580 *	0.359
12 month	0.108 **	0.400	0.642 **	0.512

^a The t test was used to compare means. Means in each row with asterisks are significantly different, * $p < 0.05$ and ** $p < 0.01$.

Necropsy

In each challenge group, one or two possums either died due to advanced disease, or were euthanased *in extremis* in the sixth week after challenge. Typical tuberculous lesions, involving lobar consolidation with central necrosis, were observed in the lungs of all challenged possums. The mass of the lung lesions was estimated from the lung weight. Macroscopic lesions were seen outside of the thoracic cavity, in the spleen, liver, mesenteric and hepatic lymph nodes, and kidneys, but there were no significant differences between treatment subgroups in the number and distribution of these lesions. In each trial except the two-month Trial B, there was a significant difference in the number and distribution of microscopic lesions between vaccinates and controls (Table 2.1).

For each subgroup, the ratio of weight change after infection to challenge weight, the ratio of lung weight to *post mortem* body weight, bacterial count for lung and spleen, and the number of microscopic lesions in body lymph nodes are shown in Table 2.1 along with the results of statistical comparisons.

Possums challenged 2 months after vaccination - Trial A: The vaccinated subgroup had significantly less severe lung lesions than the control subgroup and the bacterial load in the spleen was also significantly less than for the control subgroup (Table 2.1). The vaccinates lost less weight than the controls but the difference was not significantly

greater than for control possums. The bacterial count in the lungs of vaccinates was not significantly different for the controls. Significantly fewer body lymph nodes contained lesions in vaccinated possums compared with the controls (Table 2.1).

Possums challenged 2 months after vaccination - Trial B: Although there vaccinates lost less weight and had lower bacterial counts in the lungs and spleen, the differences were not significant (Table 2.1)

Possums challenged 6 months after vaccination: The change in body weight between challenge and death for vaccinated possums was significantly less than for the controls (Table 2.1). There was a significant difference in the progression of disease between groups. The mean bacterial count in the spleens of vaccinated possums was significantly less than for the control possums. There were no significant differences between treatment subgroups in mean bacterial lung counts and mean lung weight. Significantly fewer body lymph nodes in vaccinated possums contained microscopic lesions compared with the controls (Table 2.1).

Possums challenged 12 months after vaccination: There was no significant difference between the vaccinated and control possums in the change in body weight, lung weights and bacterial counts in lungs and spleen (Table 2.1). Significantly fewer body lymph nodes in vaccinated possums contained microscopic lesions compared with the controls (Table 2.1).

DISCUSSION

In the present study a protective effect of BCG vaccine in possums was demonstrated at two, six and 12 months after vaccination. The three subgroups that were vaccinated at the one time (December 1997) showed similar early responses to vaccination, as measured by the lymphocyte proliferation assay four weeks after vaccination. The differences seen in the response of vaccinated possums to experimental infection over the 12 months of the study were consistent with the waning of protective immunity with time following a single vaccination. There was no indication of differences in the primary response to vaccination. The waning of the effect of vaccination was seen in the decreasing differences between vaccinates and controls across the range of

measures of protection. At 12 months the only detectable difference remaining between vaccinates and controls was in the distribution of lesions. Factors that could have contributed to the decline in the observed level of protective immunity were season, although both the 2 month and the 12 month challenges were done in February, and the increasing age of the possums. However, the most plausible explanation for the increased susceptibility to challenge is the waning of the protective immunity. Middlebrook (1961) reported that 23 months after BCG aerosol vaccination of guinea pigs a significant level of protective immunity remained, although the level of immunity had declined

The protective effect of BCG intranasal aerosol vaccination of possums was exhibited as a marked reduction in disease severity following challenge with virulent *M.bovis* and was in keeping with the effects reported by Aldwell et al (1995a). Possums challenged two months after vaccination (Trial A) had less severe lung lesions, and lung lesions that contained fewer bacteria than controls. There was less internal dissemination of infection to the spleen and other tissues, and vaccinates lost less weight as a result of challenge. In all trials except 2-month Trial B, vaccination limited dissemination of infection from the thoracic cavity to body lymph nodes. In the 6-month and 12-month trials vaccination also limited the development of lesions in the abdominal cavity. In humans and guinea pigs BCG vaccination limits the internal dissemination of experimental infections (Lagaranderie et al 1993; Rodrigues et al., 1993). In guinea pigs BCG prevents the spread of infection from the initial site of implantation (Fok et al., 1976, Legaranderie et al., 1993). Sutherland and Lindgren (1979) showed also that BCG did not protect against initiation of infection, that is, implantation and persistence of virulent infection, but did protect against the development of disease.

We were able to demonstrate that the vaccine significantly influenced the response to challenge, but it was not always the same measurement where significant effects were seen. Nevertheless, the pattern of suppression of the disease process is evident in the findings from this set of studies. It was not possible to directly compare the results of challenge at the different time points because of changes in the possums over time, the possible effect of season and differing challenge doses

One vaccinated group did not show a protective response matching that of the remaining groups. Vaccination failed to protect the two-month Trial B possums, the only group vaccinated on a different occasion and with a different BCG suspension. The vaccine suspension used with these possums contained essentially the same concentration of BCG, but vaccination elicited poor and variable LPA responses. After challenge the vaccinated group was not significantly different from the controls in any measure except the concentration of *M.bovis* in lung tissue. We cannot explain definitively why vaccination of this group failed. Possible explanations, supported by the LPA data, are that the vaccine had poor immunogenicity due to reduced viability of the organisms or some similar explanation, or less plausibly, that these animals were in some way immunosuppressed as a group and could not respond to the vaccine.

Intratracheal infection resulted in severe, fulminant infection and a rapid disease course, with some possums dying six weeks after infection. The challenge procedure led to severe lung lesions that mimicked those seen in naturally infected wild possums late in the course of the disease, but did not produce the degree of early peripheral lymph node involvement which is a characteristic feature of the natural disease. Moreover, the time course of the resulting disease did not reflect that seen in wild possums, where the mean survival time from the appearance of clinical signs was 6 months (Jackson 1995). The results of intratracheal challenge in this study are consistent with those reported in earlier similar studies (Buddle et al 1994). All challenged possums developed severe lung abscessation and the infection spread to the spleen in the majority of possums. The rapid and somewhat atypical progression of the disease after intratracheal infection of captive possums may be the result of either different pathogenesis, the influence of maintaining the possums in enclosures, or the interaction between the two. Challenge by "natural" transmission, from other diseased possums, or from a procedure where infection progresses more slowly, may be necessary to determine vaccine efficacy in captive possums. Alternatively, the vaccine could be assessed in a wild possum population where tuberculosis is endemic.

Vaccination has the potential to benefit the control of tuberculosis in wild possum populations. Possums in the terminal stages of tuberculosis are the most significant source of infection for cattle and deer (Paterson and Morris 1995; Sauter and Morris 1995). We have shown that BCG vaccination decreases the rate of disease

development, and slows the internal dissemination of infection. Vaccinated animals are therefore less likely to reach the highly infectious late stages of the disease before they die of some other cause. Death from a cause other than tuberculosis is an advantage in that it would avoid the period of exposure of cattle and deer from possums terminally ill from tuberculosis.

All strategies using vaccination for the control of tuberculosis in wild possum populations will require the population to be revaccinated at regular intervals. Revaccination will be needed to protect susceptible members of the population: possums not vaccinated in an earlier round of vaccination, immigrants to the area and possums born since the previous round of vaccinations. Re-vaccination may also be required to boost waning immunity. If a cost-effective method of delivering vaccine can be developed, such as by possums self-administering an aerosol vaccine from a specially designed bait-station, vaccination could be a significant additional control measure to combat tuberculosis in possums. The approach may also be applicable to other wildlife reservoirs of tuberculosis, such as badgers and deer.

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Chapter 3

Vaccination of the brushtail possum (*Trichosurus vulpecula*) against *Mycobacterium bovis* infection with bacille Calmette- Guérin: the response to multiple doses *

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ABSTRACT:

In New Zealand the brushtail possum (*Trichosurus vulpecula*) is the principal wildlife vector of bovine tuberculosis. Control of infected possum populations contributes to the control of tuberculosis in domestic livestock. Vaccination is potentially a complementary strategy to population control, but to be cost-effective, administration of the vaccine to possums would need to be from an appropriately designed automatic vaccinator. Possums themselves would activate the vaccinator so that it would deliver an aerosol spray of vaccine. There would be no direct way to prevent possums receiving multiple doses of vaccine. This study examined the effect on protective immunity of repeated vaccination. Captive possums were vaccinated with BCG strain 1173P2 either 12 times at weekly intervals, twice at six-weekly intervals, or once. Vaccination was by a combination of intranasal aerosol and conjunctival instillation. Eight weeks after the last dose of vaccine, all possums were challenged intratracheally with *Mycobacterium bovis* strain 83/6235. Vaccination induced a significant immune response as measured by the lymphocyte proliferation assay. A significant level of protection, as measured by the response to challenge, developed in all the vaccinated possum groups, and protection was greater in the group vaccinated 12 times. It was concluded that protection may be enhanced if vaccinations were repeated at short intervals (weekly) but no benefit or detriment resulted from revaccination after longer intervals (one to two months).

Keywords: brushtail possum, *Trichosurus vulpecula*, bacille Calmette-Guérin, vaccination, bovine tuberculosis, *Mycobacterium bovis*.

INTRODUCTION

Wild animal populations infected with *Mycobacterium bovis* may act as reservoirs of infection for domestic animals, other wild animal species and humans. Wildlife reservoirs of bovine tuberculosis are becoming a worldwide problem. They have been identified in cervids in North America (Schmitt et al., 1997), badgers in the United Kingdom and the Republic of Ireland (O'Reilly and Daborn, 1995; Hughes et al., 1996), African buffalo in South Africa (Keet et al., 2000), and brushtail possums in

New Zealand (Morris et al., 1994). Vaccination of infected wild animal populations may be the preferred option for control where the animals have high economic or high conservation value (Hughes et al., 1996), and it may form part of an integrated control program for other species.

The brushtail possum (*Trichosurus vulpecula*), introduced to New Zealand in the middle of the nineteenth century, is now a major pest species. It causes ecological damage and is the major wildlife reservoir of *M. bovis* (Morris et al., 1995). Tuberculosis is endemic in possums over a quarter of the country's land area (Animal Health Board, 2000). Infected populations are controlled by trapping and poisoning to decrease the prevalence of disease and the risk of transmission of infection to domestic stock (Caley et al., 1999). However, population control programs are costly and without continued culling populations rapidly recover (Coleman and Livingstone, 2000). Even with intensive culling localised clusters of infection may not be eliminated and as the population recovers, disease prevalence increases again. Additional strategies are required to control tuberculosis in possums and vaccination is a promising option (Buddle et al., 2000).

Vaccination with the attenuated strain of *M. bovis*, bacille Calmette-Guérin (BCG), has been shown to significantly decrease the severity of disease and the dissemination of infection following experimental challenge (Aldwell et al., 1995a and 1995b; Buddle et al., 1997; Comer et al., 2001). Possums have been vaccinated successfully by intranasal aerosol (Aldwell et al., 1995a), intratracheal instillation (Aldwell et al., 1995b), intraduodenal instillation (Buddle et al., 1997) and subcutaneous inoculation (Aldwell et al., 1995a). After intranasal aerosol vaccination, a protective response (as measured by reduction in disease severity) was still detectable after 12 months (Comer et al., 2001).

For vaccination of wild populations to be cost-effective, possums will probably be vaccinated by self-administration of an aerosol vaccine from an appropriately designed automatic vaccinator or by oral bait vaccine. With self-administration of vaccine there would be no immediate control over the frequency with which individual possums might be re-exposed to the vaccine. Possums might be exposed to multiple doses of vaccine over a short period of time or repeated doses over several months. The

objective of the present study was to examine the effect on protection of multiple doses of BCG, administered over several months.

MATERIALS AND METHODS

Experimental design

Possums were vaccinated either 12 times at weekly intervals, twice with six weeks between doses or once. The response of vaccinated possums was compared with that of an unvaccinated control group. The response to vaccination was monitored using the lymphocyte proliferation assay and the level of protection was determined by comparing the severity of disease between treatment groups after intratracheal challenge with *M. bovis*.

Animals

Forty adult male possums weighing 1.7 - 4.2 kg were trapped in an area free of possum tuberculosis. The possums were acclimatised to captivity for three to six weeks prior to experimentation. Possums were allocated to one of four treatment groups based on body weight, such that the mean body weight of each group was similar.

Each treatment group was housed communally in a large wire-mesh-covered pen. To minimise the risk of BCG being transmitted between groups, they were housed in separate pens. After challenge the possums were held in two pens, with one half of each group in each pen.

Throughout the study the possums were examined visually each day for signs of ill health, especially for signs of adverse reactions to vaccination. For handling, the possums were sedated with 100 - 150 mg ketamine hydrochloride (Parnell Laboratories, East Tamaki, New Zealand) given by intramuscular injection. For administration of the challenge inoculum, possums were sedated with 70 mg of ketamine then anaesthetized with 12 mg of Saffan (Pet Elite Ltd, Lower Hutt, New Zealand) administered by intramuscular injection. Possums were euthanased while under ketamine sedation by the intraperitoneal injection of pentobarbitone.

Vaccination

BCG Pasteur strain 1173P2 was grown to mid-log phase in Tween albumin broth (TAB) and the number of organisms estimated from the turbidity of the culture, with dilutions made in sterile TAB (Aldwell et al., 1995b). The number of colony forming units (cfu) was determined by plating 10 fold dilutions on modified Middlebrook 7H11 agar. The vaccination suspension contained approximately 1×10^8 cfu per ml. A single batch of vaccine was grown, divided into 10 ml aliquots and stored at -80°C before use.

The vaccine was administered as a metered aerosol spray of 100 μl (Valois Spray atomisers, Douglas Pharmaceutical, Auckland) directed to each nostril from a distance of 1 - 2 cm and by instilling into the conjunctival sac of each eye a drop (50 μl) of suspension. Conjunctival vaccination has been shown to be as effective as intranasal aerosol (Comer unpublished observations) and was included as the spray from a vaccinator may deliver vaccine to both the nasal cavity and the conjunctiva.

Possums in Group 1 were vaccinated 12 times at weekly intervals, those in Group 2 were vaccinated twice, in Weeks 6 and 12, and those in Group 3 were vaccinated once, in Week 12. The final vaccination for Groups 1 and 2, and the vaccination of Group 3 was performed on the same day. Group 4 were the unvaccinated controls.

For the first, sixth and twelfth doses of vaccine for Group 1, and for all the vaccinations of Groups 2 and 3, the possums were sedated and vaccinated by both the intranasal and conjunctival routes. For the remaining vaccinations of Group 1, the vaccine was administered by the intranasal route only, with the possum fully conscious but restrained.

Lymphocyte proliferation assay (LPA)

The immunological response to vaccination was monitored with a lymphocyte proliferation assay at one, six and 12 weeks into the vaccination program, four weeks after the last vaccination, at challenge, and four weeks after challenge. The proliferation assay was performed as previously described (Cooke et al., 1999). Briefly, 1 ml of blood was added to 50 ml of lysing buffer containing 0.17 M Tris and 0.16 M NH_4Cl , pH 7.2 and incubated at 37°C for 10 min. The cell suspension was centrifuged at 350 g

for 10 min, resuspended and washed twice in PBS. The cells were finally resuspended in RPMI 1640, containing 2% normal possum serum, 2 mM glutamine and antibiotics. To each well of a sterile cell culture microtitre plate was added 200 μ l of cell suspension containing approximately 1×10^6 mononuclear cells/ml. Cells were cultured with specific antigen bovine purified protein derivative (PPD; CSL Ltd, Melbourne, Australia), 60 μ g/ml final concentration, or a non-specific mitogen Concanavalin A (Con-A; Sigma, St Louis, MO, USA), 5 μ g/ml final concentration. Concanavalin A was included to demonstrate the viability of the lymphocytes in the blood samples. For the unstimulated control wells, 50 μ l of RPMI 1640 medium was added. Assay conditions, harvesting and β -scintillation counting were as described previously (Cooke et al., 1999). The response to Con A was in the range of 50,000 to 500,000 counts per minute (cpm). The stimulation index (SI) was calculated as the mean response to bovine PPD divided by the mean of the unstimulated control wells.

Challenge

The vaccinated possums were challenged eight weeks after the last vaccination, in Week 20, at the same time that the control possums were challenged. The challenge suspension was prepared from *M. bovis* strain 83/6235, originally isolated from a naturally infected possum (Buddle et al., 1994). The preparation of the challenge inoculum was as described previously (Aldwell et al., 1995b). For inoculation a 1.5 mm external diameter plastic cannula was passed down the trachea until the end was below the bifurcation. When it was in place, 200 μ l of the *M. bovis* suspension, containing approximately 50 cfu, was instilled into the lungs and the cannula was flushed with 200 μ l of sterile saline. The possums were then placed in left lateral recumbancy to recover.

Necropsy

All possums were examined *post mortem* using a standardised procedure. Possums surviving to the ninth week after challenge were euthanased. *Post mortem* body weight was recorded, as was lung weight. At necropsy the distribution of macroscopic lesions in the lungs and other tissues and organs were recorded. Lung weight was taken as a measure of the severity of lung lesions, while the number of macroscopic lesions in the spleen and the colony count from the spleen were taken as measures of disease

progression. The ventral third of the spleen and a sample of a typical lung lesion (approximately 1 g) were collected from each possum for bacteriological examination.

Tissues were collected for histological examination and preserved in 10% formalin. The following paired lymph nodes were collected: mandibular, parotid, retropharyngeal, caudal superficial cervical, deep axillary, superficial axillary, inguinal, iliac and tracheo-bronchial; and the following single or groups of lymph nodes: hepatic, gastric and mesenteric. Other tissues collected for histological examination were the tonsils, liver, lung, spleen, kidneys and adrenal glands. For examination tissues were embedded in paraffin, sectioned at 3 μm , and stained with both haematoxylin and eosin, and by the Ziehl-Neelsen method. Histological examination consisted of detection of granulomas that contained acid-fast bacteria (AFB). A tissue was scored as positive if it contained one or more tuberculous granulomas.

Bacteriology

Bacteriological examination was as described by Corner et al. (1995) and isolates of *M. bovis* were identified as described by de Lisle and Havill (1985). For the enumeration of mycobacteria from lung and spleen, appropriate dilutions of homogenised tissue were made in TAB. Plates of modified 7H11 agar medium were inoculated with 0.1 ml of a 1/10 and 1/1000 dilution.

Statistical analysis

The counts per minute in the LPA were \log_{10} transformed to achieve normality. ANOVA was used to compare LPA responses at each bleed, the proportional change in body weight (kg/kg) between challenge and necropsy and lung weight at *post mortem* as a proportion of body weight at challenge (g/kg). Statistical significance for the ANOVA was set at $p < 0.05$. The Kruskal-Wallis test was used to compare group medians for lung bacterial counts and spleen bacterial counts. Where a significant difference was found with the Kruskal-Wallis test, four pairwise comparisons were made using the Mann-Whitney U test and the significance level adjusted for the multiple comparisons ($p < 0.01$). For analysis, bacterial counts were \log_{10} transformed.

For the analysis of the difference in the frequency and distribution of microscopic lesions, each tissue was coded in a binary fashion according to the presence or absence

of lesions (1.0 respectively). A random effects, binary, logistic regression analysis was then performed to determine the effect of treatment on the outcome while taking into account the clustering of tissue samples at the possum level. Model fit was checked using the deviance and extra dispersion values.

Statistical analyses were performed in SPSS (version 9.0, www.spss.com.) and SAS (version 8.0; www.sas.com.). All comparisons were performed at $\alpha = 0.05$.

RESULTS

Clinical observations

There were no clinical effects following vaccination in any possum. After challenge, clinical signs were seen in possums with advanced tuberculosis. Wasting was the most common sign, with diarrhoea occasionally seen. No palpable lesions were detected in any possum when examined four weeks after challenge, or at necropsy.

Four possums died due to advanced tuberculosis. One possum from the control group (Group 4) and one from Group 3 died in Week 27 (seven weeks after challenge), while possum from Group 2 and one from Group 1 died in Week 28. The remaining possums were euthanased in Week 29.

Lymphocyte Proliferation Assay

After vaccination the possums responded strongly to bovine PPD in the LPA (Figure 3.1). At Week 6 the bovine cpm for Group 1, which at that time had been vaccinated 5 times, was significantly greater than for the other three groups (Figure 3.1). At Week 12, Group 1 (vaccinated 11 times) and Group 2 (vaccinated once), had significantly greater responses than the other two groups, but the response of Groups 1 and 2 were not significantly different. By Week 16, the response of Group 1 (vaccinated 12 times), Group 2 (vaccinated twice) and Group 3 (vaccinated once) were each significantly greater than Group 4 (control) but the three vaccinated groups were not significantly different from each other. When challenged at Week 20, the responses of the vaccinated groups had fallen but all were significantly greater than Group 4, and Group 3 was significantly greater than Group 1. At four weeks after challenge all groups showed a marked increase in their LPA responses but Group 1 was significantly less than Group 4.

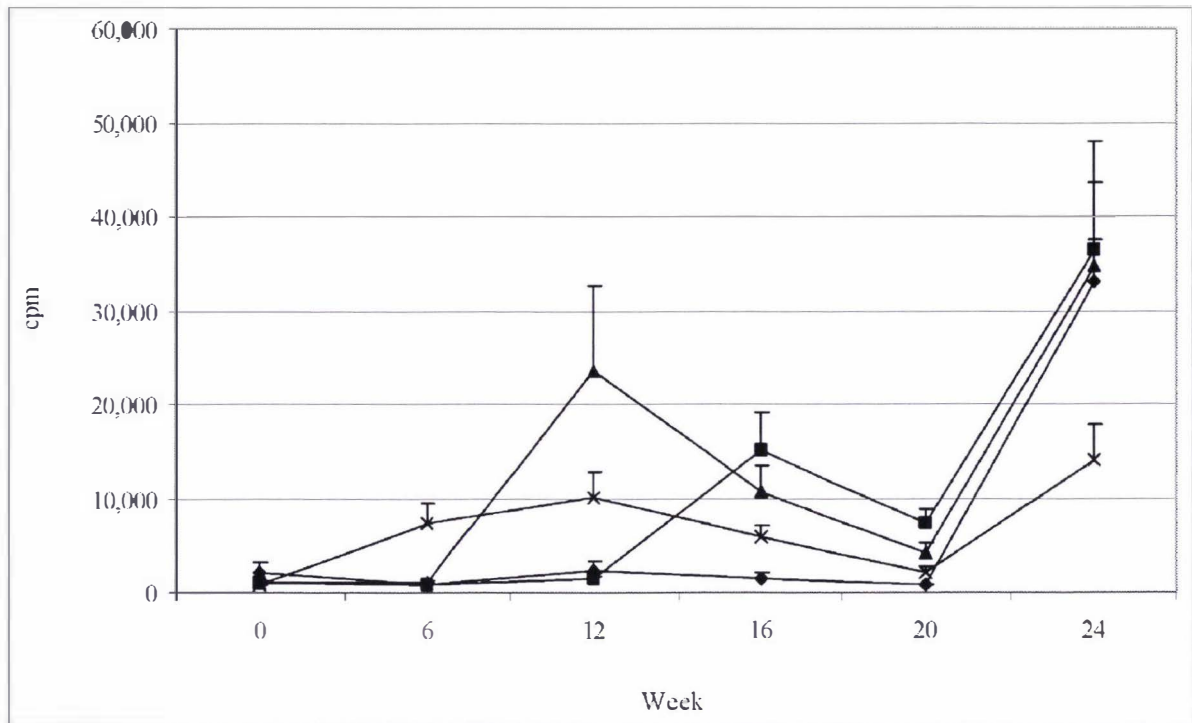


Figure 3.1

Response of possums to bovine purified protein derivative in the lymphocyte proliferation assay (counts per minute; cpm) after vaccination with bacille Calmette-Guérin and challenge with *Mycobacterium bovis*: Possums were vaccinated either 12 times at weekly intervals (Group 1, x), twice six weeks apart (Group 2, ▲), once (Group 3 ■) or were unvaccinated (Group 4, ◆). All possums were challenged in Week 20 and examined *post mortem* in Week 29. Bar indicates SE.

Necropsy

A summary of the necropsy findings are shown in Figure 3.2. All possums had macroscopic lung lesions. Lung weight, as a proportion of body weight at challenge, in Group 1 was significantly less than each of the other three groups (Figure 3.2A). The other two vaccinated groups, Group 2 and 3, were also significantly less than Group 4, although not different from each other. Possums in Group 1, 2 and 3 had significantly fewer microscopic lesions than did those in Group 4, but these three groups were not significantly different from each other (Figure 3.2B). The proportional change in body weight (change in body weight between challenge and necropsy divided by the body

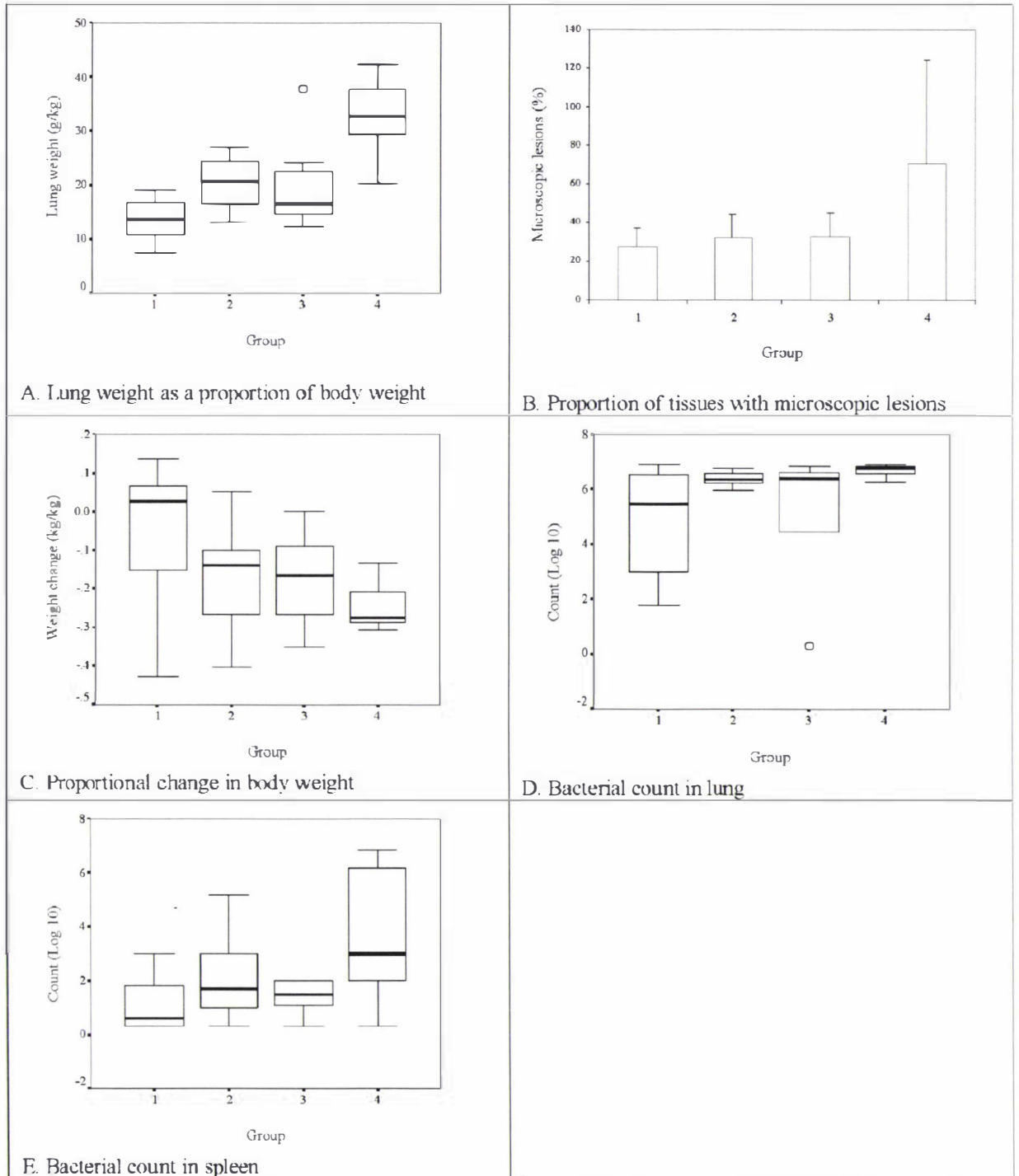


Figure 3.2

Response of possums to challenge with *Mycobacterium bovis*: Possums were vaccinated 12 times at weekly intervals (Group 1), twice six weeks apart (Group 2), once (Group 3) or remained unvaccinated (Group 4). All possums were challenged in Week 20 and examined post mortem in Week 29. The box and whisker plots show the

mean (broad central line), the interquartile range (box), the range of values (bars) and outliers (circles). Bar in Figure 3.2B indicates SE.

A. Lung weight as a proportion of body weight: lung weight (g) divided by body weight at challenge (kg).

B. Number of tissues with microscopic lesions. Bar indicates +95% confidence interval.

C. Proportional change in body weight: body weight at challenge minus body at necropsy divided by body weight at challenge.

D. Lung bacterial count (\log_{10} counts/g).

E. Spleen bacterial count (\log_{10} counts/g).

weight at challenge; Figure 3.2C) in Group 1 was significantly less than in the other three groups. Although Groups 2 and 3 lost less weight than Group 4, the differences were not significant.

Each of the vaccinated groups had a lower mean bacterial counts in the lungs than Group 4 (Figure 3.2D) but only the difference between Groups 1 and 4 were significant. All the vaccinated groups had lower bacterial counts in the spleen than Group 4 (Figure 3.2E) but only Groups 1 and 3 had significantly lower counts than the control group.

DISCUSSION

A protective effect of BCG vaccination was demonstrated with each of the vaccination regimes. The protective effect, seen as less severe lung lesions and fewer microscopic lesions, was observed in each of the vaccinated groups. In previous BCG vaccine studies in possums, lung weight has been used to assess the response to challenge and differences in lung weight between treatment groups was used to define protection (Aldwell et al., 1975a; Buddle et al., 1997; Comer et al., 2001). In the current study the lung weight of the possums vaccinated 12 times was significantly less than those vaccinated once or twice. A visual appraisal of Figure 3.2 shows that the group vaccinated 12 times was consistently less affected by challenge than the unvaccinated group, and the difference was statistically for all variables. Groups 2 and 3 were always intermediate between Groups 1 and 4, but differences were not all statistically significant.

Revaccination after a period of six weeks had no beneficial and no deleterious effects on the level of protection induced by a single dose of vaccine. This has also been the finding in human subjects where re-vaccination provided no additional protection in the prevention of clinical cases of tuberculosis (Karonga Prevention Trial Group, 1996; Reider, 1996). Also revaccination did not extend the duration of protection and did not lead to adverse effects (Cohn, 1997). Our findings do not agree with those of Griffin et al. (1999) who demonstrated increased protection against disease, but not against infection, in deer given two doses of BCG vaccine two months apart.

Although the protective effect of BCG was modest, it should be noted that the experimental challenge is particularly severe, with all possums developing severe tuberculous pneumonia by 6 - 8 weeks. In naturally infected possum populations the prevalence of disease estimated from cross-sectional surveys is typically in the range 1 - 10% (Coleman and Caley, 2000) and the progression of the disease is considerably slower (Jackson, 1995). The experimental challenge used in this study is relevant for identifying the best options for field evaluation. A study in a wild possum population has shown BCG vaccine had relatively high efficacy. Over a two-year period four *M. bovis* infected possums were identified in 149 BCG-vaccinated possums compared with 13 infected among 151 unvaccinated possums (Corner unpublished observations).

The LPA response increased markedly following BCG vaccination, but was not boosted by revaccination. This was seen in both groups that received multiple doses of vaccine. At challenge all the vaccinated groups had a significantly greater LPA response than the controls and the response of Group 3, vaccinated only once 4 weeks earlier, was greater than the other two vaccinated groups. There was a difference between groups four weeks after challenge, with the group vaccinated 12 times showing the lowest response. That is, the group that had the greatest level of protection showed the lowest response to challenge. Buddle et al. (1995) reported a similar pattern in cattle, where those protected by vaccination with BCG had a lower response following challenge.

Our findings that revaccination will at best enhance protection and at worse have no deleterious effects, means that simple strategies can be developed to incorporate vaccination into the control of wild possum tuberculosis. Population dynamics, cost-

effectiveness, and the need to achieve a high level of exposure within the population will determine the frequency of vaccination. These strategies can be developed without concern for over exposure of individuals to vaccine. Other than to discourage wastage, vaccine dispensers will not have to incorporate design features that discourage possums from repeatedly using them.

The results of this study should remove fears that repeated exposure to vaccine, which may occur when wild possums administer vaccine by self activating an aerosol spray of vaccine, will have adverse effects on the level of protection. Repeated exposure to vaccine at short intervals over several weeks increased protection and repeating vaccination after an interval of six weeks did not change the protection induced by the first dose. These results may be applicable to a wide range of wild and domestic animal species. Tuberculosis is endemic in one or more wild animal species in several countries and the disease is a threat to their conservation, as well as a threat to domestic animals and humans. Vaccination could be usefully incorporated into the control of tuberculosis wherever animals of high economic, social or conservation value are involved and test and slaughter or culling programs are not applicable.

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Chapter 4

Conjunctival vaccination of the brushtail possum (*Trichosurus vulpecula*) with bacille Calmette-Guérin *

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ABSTRACT:

The brushtail possum (*Trichosurus vulpecula*) serves as the principal wildlife reservoir of *Mycobacterium bovis* in New Zealand. The current procedure for control of disease in these populations is by culling. Vaccination of possums against *M. bovis* infection is potentially an alternative or complementary control procedure. Vaccine could be delivered to possums using an aerosol vaccinator and the current study investigates the effectiveness of vaccination via the conjunctival route. Nine adult male brushtail possums were vaccinated by the instillation of approximately 1.5×10^5 colony forming units (cfu) of bacille Calmette-Guérin (BCG) strain Pasteur 1173P2 into the conjunctival sac of each eye. There was no conjunctival inflammation or other adverse reactions to the administration of the vaccine. By 8 weeks after vaccination there was a significant immune response to *M. bovis* antigens, as measured by the lymphocyte proliferation assay. At that time the vaccinated possums and 10 unvaccinated control possums were challenged by intra-tracheal instillation of approximately 50 colony forming units of *M. bovis*. Conjunctival vaccination induced a significant level of protection. Although all possums had disease, the vaccinates had significantly less severe lung disease and less dissemination of infection to the spleen. BCG vaccine sprayed into the eyes and nose of a possum using an aerosol vaccinator could be effective in preventing the spread of this disease in wild possum populations.

INTRODUCTION

Bovine tuberculosis in wild animals that then act as a reservoir of infection for domestic animals, other wild animal species or humans, is becoming a world wide problem. Significant wild animal reservoirs have been identified in cervids in North America (Schmitt et al., 1997), in badgers in England and Ireland (O'Reilly and Daborn, 1995; Hughes et al., 1996), in Cape buffalo in South Africa (O'Reilly and Daborn, 1995), and brushtail possum in New Zealand (Morris et al., 1994). Vaccination of wild animals may be the only alternative to culling as a means of controlling infection in wild populations. Vaccination would be the preferred option for wild animal species of high economic or high conservation value (Hughes et al., 1996).

In New Zealand, the introduced Australian brushtail possum (*Trichosurus vulpecula*) is the most significant wildlife reservoir of *Mycobacterium bovis* for domestic stock and other wildlife species (Morris et al., 1994; Morris and Pfeiffer, 1995). Infection is endemic in possum populations in over a quarter of the country's land area (O'Neill and Pharo, 1995). To decrease the prevalence and incidence of tuberculosis in possums, and to interrupt transmission of infection to domestic stock, possum populations are controlled by trapping and poisoning programs (Caley et al., 1999). A significant decrease in the transmission to cattle and deer can be achieved by these procedures (Caley et al., 1999). Control of possum numbers by population reduction is costly and an unending process. Without continued pressure populations rapidly recover and disease prevalence rates increase and may return to pre-control levels in two years (Corner unpublished).

Possoms are controlled in and around areas where tuberculosis is endemic, to contain infection, and prevent spread to adjoining populations. The depopulated areas adjoining endemic areas are called buffer zones. The width of the buffer zones are based on the dispersal behaviour of juvenile possums (Barlow, 2000) and require annual or biennial maintenance (Coleman and Livingstone, 2000). Vaccination of possums with *M. bovis* strain bacillus Calmette-Guérin (BCG) has been shown in cage trials to induce significant levels of protection and so vaccination is potentially an alternative control strategy (Morris and Pfeiffer, 1995). Buffer zones where the possum populations are vaccinated may have advantages over depopulated buffer zones.

In BCG vaccine research with captive possums a number of different routes of administration have been trialed successfully. Possums have been vaccinated by intranasal aerosol and subcutaneous inoculation (Aldwell et al., 1995a), intra-tracheal (Aldwell et al., 1995b) and intra-duodenal instillation (Buddle et al., 1997). Oral administration of vaccine was not effective probably due to the bacteria being killed by gastric secretions (Aldwell et al., 1995b). After being challenged with *M. bovis*, vaccinated possums develop less severe disease than unvaccinated possums. Following a single intranasal aerosol dose of BCG, the protective effect was demonstrable for up to 12 months (Corner et al., 2001).

The routes of administration of BCG to wild possums are limited to aerosol and oral administration are limited by their free ranging nature, and the animal's willingness to

investigate novel objects in their environment. Intranasal aerosol vaccination of possums is very effective. When possums were vaccinated by intranasal aerosol, a strong immune response was obtained, and the effect was long lasting. Oral vaccination, which has not yet been successful, may be considered if a suitable encapsulation system to protect the bacteria, can be developed.

The potential of aerosol vaccine is not restricted only to wild possums, but could be applied to tuberculosis vaccines of domesticated and wild animals. Vaccination could be used to control the disease in wild animal populations, avoiding the need for broad scale population control, the only alternative currently available. In addition, other types of live bacterial vaccines, viral vaccines or even subunit vaccines that are effective when applied to mucous membranes, could all be delivered using similar technology.

Aerosol vaccination of possums with BCG could be achieved using an appropriately designed vaccinator-bait station. Possums will investigate novel objects in their environment. Utilising their inquisitive nature, a possum-activated vaccinator-bait station that sprays vaccine into a possum's face, could be designed. Greater flexibility in the design of the vaccinator would be available, and a greater probability of delivering an immunising dose would be achieved, if the vaccine were sprayed onto the possum's face, into the eyes as well as into the external nares. However, nothing is known about the clinical and immunological effects of administering BCG vaccine to the conjunctiva.

The study reported here examined the efficacy of BCG vaccination when the vaccine suspension was delivered to the conjunctiva.

MATERIALS AND METHODS

Animals

Nineteen adult male possums weighing 1.9 kg to 3.8 kg were trapped in a region free of wildlife tuberculosis. The possums were acclimatised to captivity for a minimum of 6 weeks prior to experimentation. The communal housing and handling of the possums were as described previously (Comer et al., 2001). Possums were allocated to the vaccinated group (n = 9) or the unvaccinated group (n = 10) alternately from a

list of all possums ranked on body weight, such that the mean weight of the two groups were similar, 2.48 kg and 2.55 kg respectively. Eight weeks after vaccination all possums were challenged by the intra-tracheal instillation of *M. bovis* (Pfeffer et al., 1994) and euthanased 7 weeks after challenge.

Possums were examined visually every day, and were weighed at vaccination, 4 and 8 weeks after vaccination, when challenged with *M. bovis*, and 4 weeks after challenge. A whole blood sample was collected into heparinized vacutainers (Becton-Dickinson, Franklin Lakes, NJ) at each of these times for the lymphocyte proliferation assay (LPA).

Possums were sedated by the intramuscular injection of 100 mg to 150 mg of ketamine hydrochloride (Parnell Laboratories, East Tamaki, New Zealand) to facilitate handling and vaccination. For intra-tracheal instillation of *M. bovis* possums were sedated with ketamine, than anaesthetised with 12 mg of Saffan (Pet Elite, Lower Hutt, New Zealand).

Vaccination

*Mycobacterium bovis*_BCG strain Pasteur 1173P2 was prepared as described by Aldwell et al. (1995b). Briefly, a suspension was grown to mid-log phase in Tween albumin broth (TAB) and the number of organisms estimated from a visual examination of the turbidity of the culture. For use, the suspension was diluted to the required concentration in sterile TAB. The number of colony forming units (cfu) in the vaccine suspension was determined by plating 10 fold dilutions on supplemented Middlebrook 7H11 agar. The BCG suspension used contained approximately 3×10^6 cfu per ml.

Possums were vaccinated by instilling 1.5×10^5 cfu (a 50 μ l drop of BCG suspension) directly into the conjunctival sac of each eye. To accommodate the drop the upper and lower eyelids were retracted and the eyeball was depressed slightly. The eye was held in this position for 30 to 60 sec after the drop was delivered, to allow time for it to be absorbed, or to drain away through the lachrymal duct. For 14 days after vaccination, during the daily visual examination of all possums, special note was taken of the condition of the conjunctivae of vaccinated possums.

Challenge

The challenge suspension was prepared from *M. bovis* strain 83/6235, originally isolated from a naturally infected possum (Buddle et al., 1994). The procedure for preparing the challenge inoculum was the same as for the BCG vaccine suspension. The *M. bovis* suspension contained approximately 2.5×10^2 cfu per ml. The vaccinated possums were challenged 8 weeks after vaccination, at the same time as the unvaccinated group. A 1.5 mm external diameter plastic cannula was passed through the mouth and down the trachea until the end lay beyond the level of the tracheal bifurcation. When in place 200 μ l of the *M. bovis* suspension (approximately 50 cfu) was instilled into the lungs and the cannula was flushed with an equal volume of sterile saline. The possums were then placed in left lateral recumbency to recover.

Necropsy

All possums were subjected to a detailed post mortem examination. Possum surviving to the 8th weeks after challenge were euthanased. Post mortem body weight was recorded, as was the weight of the lungs. During the necropsy, the distribution of gross lesions in the lungs, and elsewhere, was recorded, along with a description of macroscopic lesions. A sample of lung lesion (approximately 2 g) and the ventral 1/3 of the spleen was collected from each animal for bacteriological examination.

Tissues for histological examination were preserved in 10% buffered formalin. The following lymph nodes were collected: mandibular, parotid, caudal cervical, deep axillary, superficial axillary, inguinal, iliac, tracheo-bronchial, hepatic, gastric, and mesenteric. Other tissues collected were the tonsils, liver, lung, spleen, kidneys and adrenal glands. For examination, tissues were embedded in paraffin, sectioned at 3 μ m, stained with haematoxylin and eosin and by the Ziehl-Neelsen method. A tuberculous lesion was defined as a granuloma containing acid-fast bacteria (AFB).

Bacteriology

Bacteriological examination of the lung and spleen for *M. bovis* was as described previously (de Lisle and Havill, 1985). The concentration of *M. bovis* in lung and spleen was determined by the appropriate dilution of homogenised tissue in TAB.

Plates of modified 7H11 agar were inoculated with 0.1 ml of a 1/10 and 1/1000 dilution of the homogenised lung tissue and 0.1 ml of undiluted and 1/100 dilution of homogenised spleen.

Lymphocyte proliferation assay (LPA)

The proliferation response of peripheral blood lymphocytes was measured using the method described by Buddle et al. (1992). Briefly, 1 ml of blood was added to 50 ml of lysing buffer containing 0.17 M Tris and 0.16 M NH_4Cl , pH 7.2 and incubated at 37°C for 10 min. The cell suspension was centrifuged at 350 g for 10 min, resuspended and washed twice in PBS. The cells were finally resuspended in RPMI 1640, containing 2% normal possum serum, 2 mM glutamine and antibiotics. To each well of a sterile cell culture microtitre plate was added 200 μl of cell suspension containing approximately 1×10^6 mononuclear cells / ml. Cells were cultured with bovine purified protein derivative (PPD; CSL Ltd, Melbourne, Australia), 60 $\mu\text{g}/\text{ml}$ final concentration, or Concanavalin A (Sigma, St Louis, MO, USA), 5 $\mu\text{g}/\text{ml}$ final concentration. For the unstimulated control wells, 50 μl of RPMI 1640 medium was added. Assay conditions, harvesting and β -scintillation counting were as described by Cooke et al. (1999). The proliferative response to bovine PPD was expressed as the difference in mean count before and at 4 and 8 weeks after vaccination.

Statistical analysis

The proportional change in body weight between challenge and necropsy, lung weight divided by body weight at necropsy, and the bacterial count for lung and spleen samples, were compared using the t test. Lung weight as a proportion of body weight at post mortem was taken as a measure of the severity of lung lesions. For analysis the bacterial counts for the lung and spleen were \log_{10} transformed. -

In the LPA, the a priori hypothesis tested was that the vaccinated group had a significantly greater response to bovine PPD than did the unvaccinated group. The change in the bovine PPD response at weeks 4 and 8 were compared using the ANOVA. The raw LPA data (counts per minute, cpm) were not normally distributed and for analysis were \log_{10} transformed.

Statistical analyses were conducted using SPSS for Windows version 9.0 (SPSS Inc., 1999).

RESULTS

Clinical Findings

No inflammatory responses were observed in the conjunctivae of possums following vaccination. Clinical signs of disease were seen following challenge with *M. bovis*. The most consistent sign was wasting, with a few possums also showing diarrhoea. In the last 2 weeks of the challenge period, the possums as a group, showed a conspicuous decrease in food intake. The possums were examined for superficial lymph node enlargement at 4 weeks after challenge and at necropsy but none were detected.

Three vaccinated possums died from tuberculosis at 40, 47 and 49 days after infection and three unvaccinated possum died at 41, 48 and 49 days. The remainder were euthanased at 50 to 51 days after infection.

Lymphocyte Proliferation Assay

The vaccinated possums responded strongly to bovine PPD in the LPA after vaccination and both vaccinated and unvaccinated possums after challenge. At week 8 after vaccination the increase in response to bovine PPD of the vaccinated group was significantly greater than in the unvaccinated group (Table 4.1, $p = 0.03$). Both the vaccinated and the unvaccinated groups showed a 1 to 2 log increase in the bovine PPD response after challenge compared with pre-vaccination levels (data not shown).

Necropsy

No significant differences were found between the body weights of vaccinated and unvaccinated possums at necropsy, nor in the change in body weight between challenge and necropsy (Table 4.2).

The mean lung weight of vaccinated possums was significantly less than for the unvaccinated possums (Table 4.2). Gross lesions of tuberculosis were seen in the lungs of all possums in both treatment groups. In the most severely affected possums the tracheo-bronchial lymph nodes were enlarged and prominent.

TABLE 4.1.

Conjunctival vaccination of possums with bacillus Calmette-Guérin: Changes in the lymphocyte proliferation response to bovine purified protein derivative at 4 and 8 weeks after vaccination (geometric mean).

Weeks after vaccination	Treatment Group	
	Vaccinates	Unvaccinated
4 wks	352 (43, 2911) ^a	103 (13, 857)
8 wks ^b	6194 (598, 63973)	175 (21, 1452)

^a Geometric means and 95% confidence limits of the mean

^b At 8 weeks after vaccination the vaccinates had a significantly greater response than at 4 weeks ($p = 0.05$, adjusted for multiple comparisons: Least Significant Difference) and to the unvaccinated possums ($p = 0.03$, adjusted for multiple comparisons: Least Significant Difference)

Gross lesions of tuberculosis were seen outside of the thoracic cavity in 8 animals. In one vaccinated possum there were lesions in the spleen and liver. Seven unvaccinated possums, had lesions in the spleen, and in 2 of these 7, there were lesions on the liver. Lesions in the spleen and liver ranged in size from 1 to 6 mm in diameter. The number of unvaccinated possums with macroscopic spleen lesions was significantly greater than for the vaccinated possums.

There was no significant difference in the distribution of microscopic tuberculous lesions between treatment groups.

Bacteriology

M. bovis was isolated from the lungs of all of the challenged possums and from the spleens of 7 vaccinates and 8 unvaccinated possums. Although the mean count of bacteria in the spleens of vaccinated animals was less than for the unvaccinated possums, the difference was not significant ($p = 0.48$; Table 4.2). The mean count of bacteria in the lungs was similar between treatment groups.

TABLE 4.2.

Conjunctival vaccination of possums with bacille Calmette-Guérin: Response to intratracheal challenge with *Mycobacterium bovis*.

Parameter	Treatment Group			
	Vaccinates (n = 9) ^a		Unvaccinated (n = 10)	
	mean	sd	mean	sd
Challenge wt	2.97	0.57	3.08	0.54
Weight change / challenge wt ^b	-0.18	0.02	-0.21	0.09
Lung wt / post mortem wt ^c	22.6 *	9.1	32.1 *	10.2
Lung bacterial count (log ₁₀ cfu) ^d	6.12	0.82	6.13	0.89
Spleen bacterial count (log ₁₀ cfu)	2.87	1.71	3.43	1.68
Proportion with macroscopic splenic lesions ^e	1/9 *		7/10 *	

^a n = number of possums in the treatment group

^b Weight change (kg) between challenge and post mortem divided by challenge weight (kg).

^c Lung weight (g) divided by post mortem weight (kg).

^d The t test was used to compare means and Fisher's exact test (two tailed) to compare differences in proportion of macroscopic lesions in the spleen. In each row, asterisks indicate comparisons that were statistically significant, $p < 0.05$.

cfu = colony forming units

^e The number of possums with macroscopic lesions in the spleen as a proportion of the possums in the group.

DISCUSSION

The instillation of a suspension of BCG into the conjunctival sac was an effective means of vaccinating possums. Conjunctival vaccination led to a strong LPA response to bovine PPD and the vaccinated possum showed a significant level of protection to challenge with *M. bovis*. The immune response following vaccination, as measured in the LPA, developed more slowly than we reported following intra-nasal aerosol vaccination, where the peak response was seen at 4 weeks after vaccination (Corner et al., 2001). Following vaccination via the conjunctival route the response to bovine PPD was higher at 8 weeks than at 4 weeks.

Although the challenge procedure resulted in all possums developing lesions of tuberculosis, a protective effect of BCG vaccination was seen. Vaccinates had less severe disease as measured by the extent of lung lesions, and vaccination retarded the dissemination of disease from the lungs to the spleen. In this regard, conjunctival vaccination was as effective as intra-nasal administration (Aldwell et al., 1995b; Corner et al., 2001).

It is possible that the BCG suspension, when placed in the conjunctival sac, led to infection of the conjunctivae, and, following drainage through the lacrimal duct, infection on the mucosa in the posterior nasal passages. Inhalation into the lungs from the nasal passages may also have occurred. After experimental conjunctival infection with *M.bovis* 90% of possums developed lung lesions (Corner, unpublished observations). It is unlikely that any BCG that was swallowed would have induced an immune response (Aldwell et al., 1995b). A systemic immune response was clearly present 8 weeks after vaccination, indicating that BCG infection had become established.

The LPA responses were slower to develop after conjunctival instillation than after intranasal aerosol indicating that infection took longer to establish. This may have been due either to fewer bacteria being retained to establish infection on the nasal mucosa, or fewer bacteria being inhaled directly into the lungs. This is consistent with our experience with conjunctival infection with *M. bovis*, where 25 times more bacteria were required to establish infection by the conjunctival route than by the intra-tracheal route (Corner, unpublished observations). Aerosol vaccination of guinea pigs is more effective than the intra-cutaneous route (Middlebrook, 1961). Effective aerosol vaccination of guinea pigs was demonstrated using very small doses, 20 cfu of BCG. Delayed type hypersensitivity was established but took more than 3 months to develop; when a dose 50 times larger was used the response developed in less than 2 months (Middlebrook, 1961).

Vaccination of possums has the potential to be of great benefit in the control of bovine tuberculosis. It has been postulated, and supported by circumstantial evidence and behavioural studies, that possums terminally-ill with tuberculosis, are the most significant source of infection for cattle and deer (Morris and Pfeiffer, 1994; Paterson and Morris, 1995; Sauter and Morris, 1995). We have shown that BCG vaccination

decreases the severity of disease and slows the progression from infection, thereby increasing the chances of a possum surviving tuberculosis and dying of other causes. Death from other causes is an advantage in protecting domestic livestock as it would avoid the risk of them being exposed to terminally-ill tuberculous possums.

Vaccination of free-ranging wild possums has shown BCG vaccine to have high efficacy and to be practical (Corner, unpublished observations). Vaccination of wild populations could interrupt not only possum-to-possum transmission but also transmission from possums to domestic livestock. It could be applied both at the individual farm level and on a regional level. Population control alone has not been effective in controlling the expansion of the areas infested with tuberculous possums (Coleman and Livingstone, 2000). Population control and vaccination could be used in combination to control the spread to new wildlife populations by creating a buffer area of immune possums.

Vaccination is potentially a useful tool for the control of tuberculosis in wild possum populations. For BCG vaccination of wild possums to be adopted, a practical, cost effective delivery system is required. The means for administering BCG to wild possums has yet to be determined. Intra-nasal, intra-tracheal and intra-duodenal administration have all been shown to induce protection in captive possums. Conjunctival administration can now be included in the range of possible methods for vaccinating wild possums. By using an aerosolised vaccine, the mucosa of the upper and lower respiratory tract and the conjunctiva would be exposed, thereby maximising the chances of producing a strong protective response.

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SECTION C

Pathogenesis And Transmission Studies

Using Captive Possums

Introduction

In the first three experiments the challenge procedure used was the intratracheal instillation of virulent *M. bovis*, a procedure developed by Buddle and colleagues at AgResearch. That procedure was used to ensure equal and reliable exposure of all possums to infection and readily established infection in all possums. However, it results in a fulminating, rapidly fatal infection, with death occurring in 6 to 8 weeks. The disease process did not mimic that seen in naturally-infected possums in the wild. In addition both vaccinated and non-vaccinated control possums developed severe disease. However, even under these severe challenge conditions an effect of vaccination was seen - less severe lung lesions, less dissemination of infection beyond the lungs, longer survival time after infection and vaccinates lost less body weight. In an attempt to better emulate the disease as seen in naturally-infected wild possums, alternative ways of infecting possums were investigated, natural transmission between possums and conjunctival infection.

We wanted to undertake a study of vaccine efficacy and tuberculosis pathogenesis in captive possums where the transmission of infection was by a natural path. By doing this we expected the disease in the "naturally" infected possums would more closely resemble that seen in the wild. We intended to use experimentally infected possums as the source of infection to other possums in the same colony. The work drew on previously unpublished research by Bryce Buddle (Experiment 1 in Chapter 6) and an experiment specifically designed and conducted to examine transmission between possums randomly allocated to treatment groups. In these experiments only close proximity of diseased and susceptible possums was considered.

It was apparent after conducting the pilot study that close proximity of infected and susceptible possums influenced the risk of transmission but the frequency and duration of social interaction would also influence the risk. We had observed that captive possums were nocturnal, that they occupied a single den during the day and that den sharing occurred frequently. The closest proximity and the longest period of contact occurred when the possums shared a den. The common airspace of the den would be an ideal vehicle for the transmission of the infectious aerosol.

In the fourth experiment (Chapter 5) we undertook a study of the den sharing behaviour of groups of captive possums. Sociometric techniques and social network analytical

procedures were used to examine the interactions, to analyse the structure of the social network, and, to explore the effects of alterations to the environment on the social network.

A natural transmission study (Chapter 6) was undertaken to establish a procedure to challenge vaccinated possums in a way akin to that which occurs in the wild. Using social network concepts and analytical techniques the social structure of two colonies was determined using den-sharing behaviour. We identified and infected the most socially active possums and studied the pattern of disease in the in-contact animals. Social network analysis (SNA) was used to obtain standardised measures of interactions. We attempted to determine the efficacy of BCG vaccination under the conditions of "natural challenge".

An experiment on conjunctival infection (Chapter 7) was conducted to establish if this route of infection would result in a disease process that would more closely reflect the time course of infection in naturally infected wild possums than other procedures. Vaccination via the conjunctival route had been successful so it was considered that experimental infection using this route would be effective. We postulated that administration of *M. bovis* by this route would lead to a slowly progressing infection where the time course and the nature of the lesions would reflect that seen in naturally infected wild possums. This could provide a better means of assessing vaccine efficacy than intratracheal infection.

Chapter 5

Examination of contagious disease transmission processes by social network analysis – using *Mycobacterium bovis* infection as an example *

ABSTRACT

While the study of disease incidence data in populations is a cornerstone of infectious disease epidemiology, there is considerable difficulty in examining the question of which animals become infected, and why. Wild brushtail possums (*Trichosurus vulpecula*) are the main source of *Mycobacterium bovis* infection for New Zealand livestock. The disease is spread principally by infectious aerosol, and therefore the extent of social interactions largely determines disease transmission. In captive possums den-sharing behaviour provided the greatest risk of tuberculosis transmission between animals. Social network analysis (SNA) concepts and methodologies were used to develop a model to describe patterns of social behaviour and to predict tuberculosis transmission. Den sharing between individual possums was used as the unit of analysis, as it represents a quantifiable opportunity for achieving close proximity between animals over extended periods. Alteration of the physical environment of the pens, such as changing the number of dens or relocating the group to a new enclosure, had little effect on social structure. The importance of the measures of social structure derived from SNA was assessed during the disease transmission study. The possums that became infected had significantly greater closeness and flow-betweenness scores than those that remained free of infection. Although simpler measures, such as the number of partners and the frequency of den sharing events, were significantly higher for the infected compared to the possums free of infection, the SNA-specific measures were more precise, able to be compared across time and between groups. SNA was a useful tool for describing and analysing the social relationships quantitatively and investigating the relationship between an individual animal's role within its social group and the risk of disease transmission.

Introduction

Although transmission of contagious diseases between animals or people by means of social interaction is the principal mechanism by which disease spread occurs, epidemiologists traditionally characterise such processes primarily by rates assessed in anonymous populations, where the detail of how animals interact is entirely lost. While this is necessary for many purposes, it would be valuable to be able to investigate which animals are more likely to transmit or receive, and which animals may, by their behaviour, be protected from infection. Social network analysis offers a way of representing an

additional dimension to disease transmission, by characterising in a quantitative way which animals are more likely to be involved in disease transmission, and why.

The brushtail possum (*Trichosurus vulpecula*) is the most important wildlife reservoir for bovine tuberculosis in New Zealand (NZ) (Morris and Pfeiffer, 1995). Culling of infected populations is used as the principal control measure, but when used alone does not eradicate the disease (Caley et al., 1999). Vaccination has been shown to induce a significant degree of protection in captive possums (Aldwell et al., 1995; Buddle et al., 1997; Corner et al., 2001) and is potentially an alternative or complementary control strategy.

Vaccine efficacy studies have been conducted using captive possums. These rely on experimental infection, and intratracheal inoculation has been the favoured procedure for challenging vaccinates (Buddle et al., 1997; Corner et al., 2001). The procedure, although reliable and repeatable, results in a fulminant, rapidly fatal disease, which is unlike that seen in naturally infected possums. The efficacy of vaccination in those studies was assessed on the relative severity of lesions in vaccinates and controls.

To better evaluate vaccine efficacy a procedure was required that would reliably achieve natural transmission among captive possums, resulting in more natural patterns of disease. However, studies have shown it is difficult to achieve natural transmission in captive possums. Aerosol and direct transmission occurred infrequently between possums held in small cages (Bolliger and Bolliger, 1948; O'Hara et al., 1976; Corner and Presidente, 1981) and no transmission occurred when possums were housed communally in a large pen (Corner et al., 2002). These studies involved random allocation of possums to the different treatments based on the assumption that the proximity resulting from sharing a cage was the main factor associated with transmission. No consideration was given to the possible confounding effect of social interactions.

An understanding of the nature of the interactions between captive possums that result in transmission of *M. bovis* was required. To achieve transmission we needed to know which possums to experimentally infect in order to maximise the likelihood of spread to other possums sharing the same pen. We also needed to ensure an equal risk of exposure of different treatment groups, to counter the confounding effect of social behaviour.

Disease transmission by aerosol or contact is predicated on social interactions. For transmission to occur, more is required than simply placing infected and susceptible animals in close proximity. Transmission of infection is the result of a complex interaction of individual animal behaviours. A social network perspective focuses on the relationships among social entities, e.g. individual animals. Social network analysis (SNA) describes the relationship patterns among interacting individuals using a well established set of precise formal definitions for the relationships (Wasserman and Faust, 1998). It provides a collection of structural models, standardised procedures and statistical methods to describe and analyse the relationships within social networks as a whole and at the level of the individual. The relationships are defined using the interactions between each social entity and all other entities in the group. SNA has its basis in graph theory, where individuals constitute nodes or vertices in a graph and the line joining vertices have defined properties that describe the connections. In disease transmission studies, the paths between individuals can imply a route of transmission. SNA has been applied to the epidemiology of HIV and AIDS (Rothenberg et al., 1996), and syphilis (Rothenberg et al., 2000). In these studies SNA was used to identify at-risk individuals and predict the outcome of infection entering social groups. SNA was seen as a potentially useful technique for understanding the network of social interactions in captive possums.

Tuberculosis in possums is a respiratory disease and inhalation of infectious aerosols is the main means of transmission (Jackson et al., 1995a). Horizontal transmission is postulated to occur during direct social interactions, such as mating, fighting, den sharing or competing for dens (Jackson, 1995). In the wild, possums sleep in dens during the day (Cowan and Clout, 2000). Dens consist of hollows in trees, clumps of vegetation, or burrows made by other animals (Cowan, 1990). Analysis of disease patterns in wild populations has indicated that transmission is strongly associated with possum denning areas (Pfeiffer, 1994; McKenzie, 1999).

The duration and nature of possum interactions are factors that influence transmission. In communally housed possums, sharing of dens occurs frequently (McLeod et al., 1997), and the closest proximity and the longest period of contact occur when possums share a den (Corner unpublished observations). The common airspace of the den was considered an ideal vehicle for the transmission of an infectious aerosol. An understanding of the

social patterns of denning behaviour in captive possums would help to identify an effective system for achieving natural transmission of *M. bovis* among captive possums.

SNA was used to develop a social model of the denning behaviour of captive possums, to investigate if changes to the environment of the holding pen had an influence on social patterns, and to validate the resulting model by comparing the association between tuberculosis transmission patterns and denning patterns.

Materials and methods

Overview

The patterns of denning behaviour were studied in 4 groups of captive possums (Table 5.1). Over a period of days the 'den' in which each possum slept during the day was recorded. Repeated observations were made on all groups to determine factors that influenced social structure and its stability. The influences examined were those of -

1. time,
2. moving the possums to a different pen,
3. increasing or decreasing the possum:den ratio, and,
4. the introduction of new members.

Tuberculosis was introduced into 2 groups to validate the use of denning patterns as a model to represent transmission patterns of *M. bovis* infection. Conventional analytical procedures were used to describe the behaviour of individual possums, and social network analysis was used to examine possum-to-possum interactions and to analyse the structure of the social network.

Study population

The study groups comprised adult male possums captured in a region free of tuberculosis in wild possums. They were held in large enclosures with floor areas of 170 m² - 240 m². Hessian sacks were hung in shelters within each pen for the possums to use as dens. Possums were individually identified with a numbered metal ear tag (National Banding Office, Wellington) and a passive transponder (ID 100, Trovan Inc.) implanted subcutaneously over the shoulders that transmitted a unique 10-digit number. The transponder was detected using a handheld detector (LID-500 Hand Held Reader, Trovan Inc.) held close, less than 20 cm, to the possum. This enabled possums to be identified in

their dens without them being disturbed. The denning location of possums was recorded each day during the observation period.

Table 5.1

The design of studies to examine the social network of groups of captive brushtail possum using observations on den-sharing behaviour.

Group	Period ^a	Duration (days)	Pen	Number of possums	Possum: den ratio
A	A1	21	1	29	1:1
	A2	21	1	29	1:0.8
	A3	21	2	28	1:1
	A4	21	2	23	1:1.6
B	B1	7	1	20	1:1
	B2	7	3	22	1:1
C	C1	14	3	24	1:0.9
	C2	14	3	24	1:0.9
D	D1	14	4	24	1:1
	D2	14	4	23	1:1

^a Group A were first observed 8 months after capture and then at 9, 10 and 12 months. Groups B, C and D were first observed 1 month after capture and again at 4 months.

Study design

Group A

The influence of time, moving the possums to a different pen and changes to the possum:den ratio were studied in Group A. Possums were observed for 4 periods, each of 21 days, at approximately monthly intervals, beginning 8 months after capture (Table 5.1). During the first period (A1) they were in Pen 1 (170 m²) with a possum:den ratio of 1:1. The number of dens was decreased between the first and second period (A2) when the 3 most frequently and the 3 least frequently used dens were removed, giving a possum:den ratio of 1:0.8. The possums were moved to a larger pen (Pen 2, 240 m²) between the second and third periods (A3) and the possum:den ratio was 1:1. Between the third and fourth periods (A4) the possum:den ratio was increased to 1:1.6 with the addition of 8

dens. During the course of the study the number of possums in Group A decreased from 29 to 23 due to the death of 6 possums that were not replaced.

Group B

The influence of time, moving the possums to a different pen and the introduction of new members were studied in Group B. Possums were observed for 2 periods of 7 days, with the first period (B1) commencing 1 month after capture and the second (B2) 4 months later. Between the observation periods 1 possum died and the group was moved from Pen 1 to Pen 3, a pen of a similar size. Three new members were added to the group 3 weeks before the second period commenced.

Group C and D

The influence of time was studied in Groups C and D, as well as the association between denning behaviour and the spread of tuberculosis. Each group was observed for two periods of 14 days. The first period (C1 and D1) was 1 month after capture and the second (C2 and D2) was approximately 4 months after the first. Tuberculosis was introduced into each colony 25 days before the beginning of the second period.

Bovine tuberculosis was introduced by experimental infection of 4 possums in each group. The possums that were experimentally infected were selected on the basis of their frequency of den sharing, and the number of other individuals with whom they had shared dens during the first observation period. The subset of 4 possums was selected such that no other combination of 4 possums interacted with more possums and on more occasions. Possums were experimentally infected by intratracheal instillation of approximately 100 colony forming units of *M. bovis* (Pfeffer et al., 1994).

Half of the remaining possums in each group were vaccinated four weeks before the introduction of infection to the colonies. To allocate possums to the vaccinated or unvaccinated groups, the possums were ranked on number of partners and frequency of interactions. From a list ordered by rank, possums were allocated alternately to either the vaccination or unvaccinated control subgroup. Vaccination was achieved by the administration of BCG suspension as an intranasal aerosol (Comer et al., 2001).

The transmission of infection was determined by post mortem examination of the in-contact possums, 22 weeks after the introduction of infection. The presence of *M. bovis* infection was determined by an extensive necropsy examination of each possum for macroscopic lesions, with confirmation by bacteriological examination of lesion material, and the histological examination of a set of 28 tissues from each possum. From possums with no macroscopic lesions, a pool of tissues consisting of lung, liver, spleen and 10 body lymph nodes was submitted for bacteriological examination.

Data analysis

Denning behaviour was analysed using two approaches. The first consisted of quantitative descriptions of den use, number of partners and number of interactions. The second consisted of calculating structural parameters using social network analysis (SNA).

Analysis of standard quantitative descriptor statistics

Den use, number of partners and frequency of interactions were standardised as a rate per 7 days to enable comparisons of data between periods of different duration. The data on the number of partners and frequency of interactions were \log_{10} transformed to achieve normality. Differences in den use, number of partners and frequency of interactions were compared between observation periods using analysis of variance. Analysis of variance was also used to compare the association between post mortem disease status of possums in Groups C and D, and den use, the number of partners and the frequency of interactions in Periods C2 and D2. Descriptive statistics were performed in SPSS (version 9.0; SPSS, Chicago, Illinois) and analysis of variance in SAS (version 8.0; SAS Institute, Cary, North Carolina). All comparisons were performed using a statistical significance level of $\alpha = 0.05$.

In each observation period possums were ranked on the number of partners and the frequency of den sharing interactions and these were compared using Spearman rank order correlation (Cramer, 1998).

Social network analysis (SNA)

Social network analysis provided a collection of structural models, standardised procedures and statistical methods to describe and analyse the relationships as defined through den sharing within a colony as a whole and at the level of the individual. The

social network was constructed as a representation of each possum's den-sharing interactions with other possums in the colony.

A social network consists of the interactions between individuals within a group and these interactions form quantifiable patterns. Social network analysis describes the social position of each individual using both direct and indirect contacts. The analysis of social structure is based on graph theory with social networks represented as a graph with individuals as vertices and interactions as lines joining the vertices (Borgatti, 1995).

Centrality is a structural attribute of each individual in a network and is a measure of their importance or prominence in the network (Degeenne and Forsé, 1999). It is a measure of the extent to which the social network revolves around each individual. A centralisation index, which summarises the scores of all the individuals in the network, is a measure of the heterogeneity of the network. The index allows comparisons to be made between different networks, or repeated observations on the same network. Closeness and flow betweenness were the two measures of centrality calculated.

Closeness is calculated as the sum of the distances between a given possum and all other possums in the network along the shortest path between them using both direct and indirect interactions (Bell et al., 1999). A direct interaction means a possum shared a den with another possum. An indirect interaction between two possums means that while there might not have been any den sharing between the two, they both might have shared a den on different occasions with a third possum and therefore had indirect contact. This concept of indirect interaction can be extended to more than one intermediate contact, resulting in an increased number of contacts between possums. The calculation of closeness (C_C) uses only dichotomous data. The total distance that possum "i" is from all other possums is:

$$C_C(n_i) = \left[\sum_{j=1}^g d(n_i, n_j) \right]^{-1}$$

where g = number of possums in the group, and $d(n_i, n_j)$ = number of lines linking possums i and j (Wasserman and Faust, 1998).

The higher the closeness score the more central is the possum in the social group. Closeness describes how readily a possum can make contact with or "reach" any of the

others in the group. For example, in the context of disease transmission, given that an infectious organism enters a social network, closeness describes for each individual, the "time-until-arrival" of the infectious organism, assuming that infection takes the shortest path (Borgatti, 1995). This means the more remote an individual possum is in the network the less likely it is that it will become infected, and also less able to infect others. On the other hand a 'central' possum will be more likely to become infected and will be very effective at spreading infection through the network.

The *closeness centralisation index* is a measure for the whole group and it quantifies closeness over all individuals in the group. It is the sum of the differences in closeness score between the most central member (highest individual closeness score) and each of the members of the group divided by the maximum closeness score possible for the group. As the index increases, individual possums in the network behave less alike, that is, they are more heterogeneous. It reaches its maximum when there is one central individual (maximum heterogeneity), and minimum when all individuals in the group are equally close (Wasserman and Faust, 1998). In the context of disease transmission, this means that in a network with a high closeness centralisation index, infecting the central individual will result in efficient disease spread, whereas infection in less central animals will be less

If social interactions are interpreted as representing a communications network, then *flow-betweenness* is a measure of the number of paths that pass through a possum along the shortest paths between all other possums (Freeman et al., 1991). It could be envisaged that an individual that lies on the shortest path regulates the flow of communications between two indirectly linked individuals (Borgatti, 1995). The prominence or importance of each possum in the network is the number of direct and indirect connections passing through it as a proportion of the total flow in the network. This normalises the score and allows comparisons between different networks (Everett and Borgatti, 1999). Flow betweenness (C_B) is calculated using the following formulae:

$$C_B(x_i) = \sum_{j < k} \frac{m_{jk}(x_i)}{m_{jk}}$$

where $m_{jk}(x_i)$ is the maximum flow that passes from x_j to x_k through possums x_i along the shortest paths and where $j < k$ and $i \neq j \neq k$ (Freeman et al., 1991).

The higher the flow betweenness score the more prominent and influential is the possum. If an individual with high flow-betweenness centrality is removed from the network, the speed and certainty of transmission from a random individual within the network to another is more affected than if an individual with a low score is removed (Borgatti, 1995).

The *flow-betweenness centralisation index* is an overall measure of the variability in the network. It is the sum of the individual scores divided by the total possible score for the group. A minimum of zero arises when all individuals have exactly the same flow-betweenness index and a maximum of one when one individual lies on the links between all others (Wasserman and Faust, 1998). In the context of disease transmission, assuming that contact is important, infection will spread quickly in a network with high flow-betweenness index because, even if an animal with low flow-betweenness score is infected first, it should not take long for another with high flow-betweenness score to become infected, and from then on infection should spread very quickly.

The social interaction that was measured in this study was den sharing. A possum sharing a den with another possum was considered a pair and each pair forming was termed an 'interaction'. Each other individual a possum shared with during an observation period was termed a 'partner', which was recorded as a dichotomous variable (0 = no recorded pairing, 1 = contact). A social network was constructed using the number of partner pairs that formed and the frequency of their interactions. Social network analysis was performed with the SNA software UCINET for Windows version 5.1.1.1 (Analytic Technologies, Harvard, Massachusetts, USA). The relations within the social network were visualised as two-dimensional graphs generated using the network graphing software KrackPlot (version 3.2; Krackhardt et al., 1994).

The closeness and flow-betweenness scores for Groups B, C and D were compared between periods using the *t* test. Non-parametric tests were used for Group A because of large differences in the variances between the observation periods. For Group A the closeness and flow betweenness scores were compared between periods firstly with the

Kruskal-Wallis test and, where significant ($p < 0.05$), pairwise comparisons were made using the Mann-Whitney U test. For the Mann-Whitney U test the significance was set at $p < 0.01$ because multiple pairwise comparisons were made. Social ranking of possums within all groups, as determined by closeness and flow-betweenness scores, were compared between observation periods using Spearman's rank order correlation.

Logistic regression analysis was used to analyse the relationship between the risk of infection and the potential risk factors of vaccination status, group and their interaction terms, as well as the social behaviour scores for closeness and flow-betweenness scores. The analysis was performed using SAS (version 8.0; SAS Institute, Cary, North Carolina).

Results

Den use and den sharing

Box and whisker plots of the number of different dens used by an individual possum, the number of other possums with which each possum shared a den (partners) and the frequency of den sharing (interactions) are shown in Figures 5.1A, 5.1B and 5.1C, respectively.

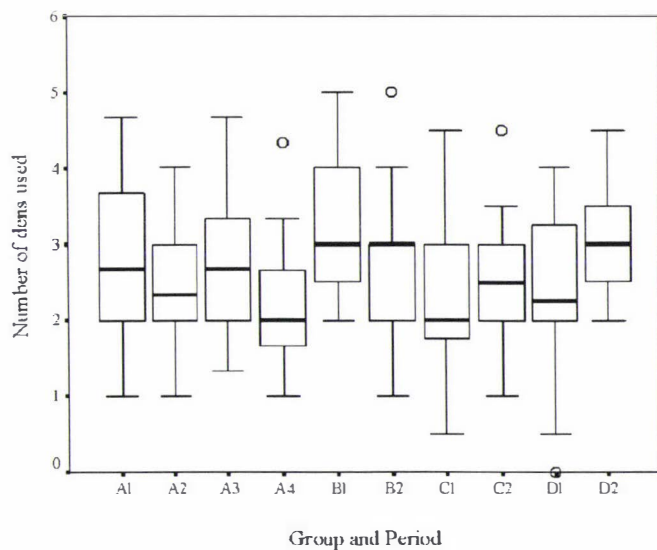


Figure. 5.1A

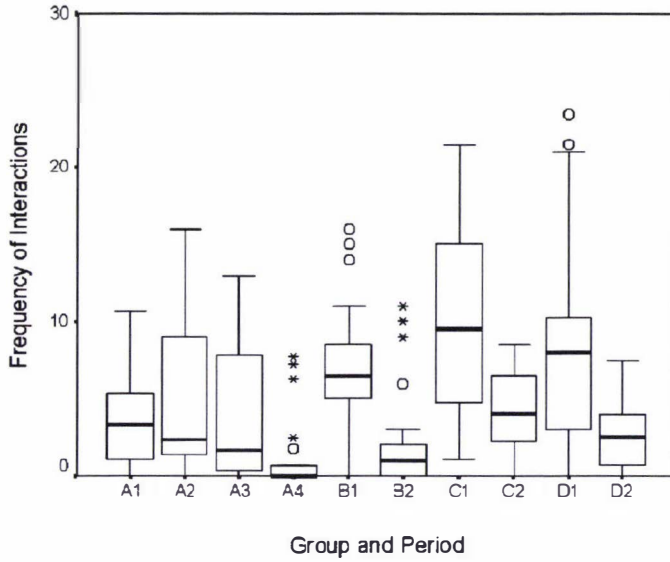


Figure. 5.1B

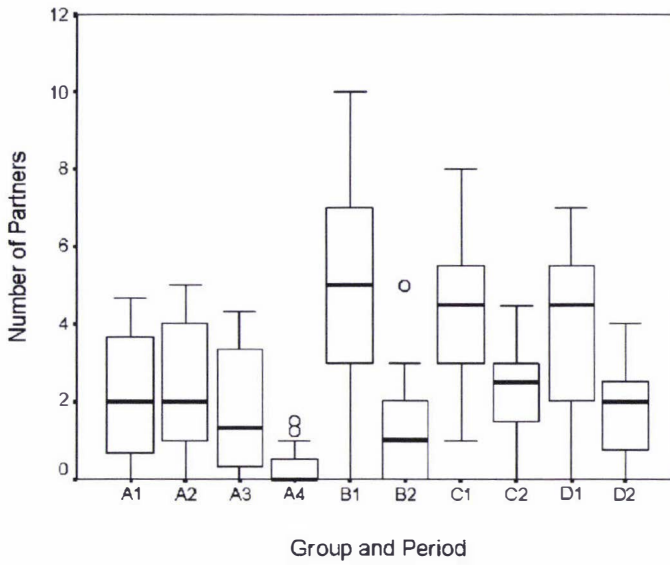


Figure. 5.1C

Figure. 5.1. Observations on the behaviour of four groups (A, B, C and D) of communally housed captive brushtail possums. Each group was observed between 2 and 4 times. For Group A the environment of the pen was changed between periods A1 and A2, A2 and A3, and A3 and A4, and for Group B, 3 new members were added to the group between Periods B1 and B2.

A. The number of dens used per 7 days for each of the four groups (A, B, C and D) at each observation period. In Period D1 one possum did not use any dens.

- B. The number of other possums in the group that each possum shared dens with (partners) per 7 days.**
- C. The number of interactions each possum had with other members of the group (interactions) per 7 days.**

Den use: There was considerable variation in the way individual possums used the available dens (Figure 5.1A). The mean number of dens used by all possums across all observation periods was 2.9 (range 0 to 4.7) per 7 days. In Group A there was a significant decline in the number of dens used in Periods A1, A2 and A3 compared to Period A4 ($p < 0.05$). In Group B there was a significant decline in the number of dens used in the second period ($p < 0.05$). There was also an increase in the number of dens used in Group C, however this was not statistically significant ($p=0.46$). In Group D there was a significant increase in the number of dens used. In Period D1, one possum did not use any dens but slept on the ground or on elevated walkways.

Partners: There was considerable variation in the number of other possums with which each possum shared a den. The median for all possums over all periods was 2 (range 0 to 10) per 7 days. The median number of partners decreased in all groups between the first and last observation periods (Figure 5.1B). There was a significant difference between observation periods for Groups B ($p < 0.001$), C ($p < 0.001$) and D ($p < 0.001$). In Group A there was no differences in the mean number of partners per possum for Periods A1, A2 and A3, however the values were significantly lower in Period A4 compared with each of the other three periods ($p < 0.05$).

Interactions: There was considerable variation in the frequency of den-sharing interactions among possums and the median decreased in all groups in successive observation periods (Figure 5.1C). The median for all possums over all periods was 3.5 (range 0 to 23). There were significant differences between the first and second periods for Groups B ($p < 0.001$), C ($p < 0.001$) and D ($p < 0.001$). In Group A there were significant decreases between Periods A1, A2 and A3 when compared with A4 ($p < 0.001$ for each comparison) and when Period A2 was compared to Period A3 ($p = 0.029$).

Social network analysis

Box and whisker plots for closeness and flow betweenness for each period are shown in Figures 5.2 and 5.3 respectively. Closeness centralisation indices and flow betweenness centralisation indices for each period are shown in Table 5.2.

Table 5.2.

The effects of time and changes to their environment on the structure of the social network of communally housed captive possums: Changes in closeness and flow-betweenness

Group	Period	Centralization index	
		Closeness	Flow-betweenness
A	A1	8	10
	A2	16	8
	A3	7	10
	A4	3	21
B	B1	16	8
	B2	5	32
C	C1	29	4
	C2	7	13
D	D1	27	4
	D2	6	14

Closeness: In Group A, closeness increased significantly when the possum:den ratio was increased (Period A1 to A2, $p < 0.0001$), decreased significantly when the ratio was decreased (Period A3 to A4, $p < 0.0001$), and also decreased significantly after the possums were moved to a new pen (Period A2 to A3, $p < 0.0001$). After they were moved to a new pen and 3 new members were introduced the measure decreased significantly in Group B ($p < 0.0001$). In Groups C and D, where the environment of the pens was left unchanged, the measure decreased significantly between periods ($p = 0.007$ and $p = 0.003$ respectively).

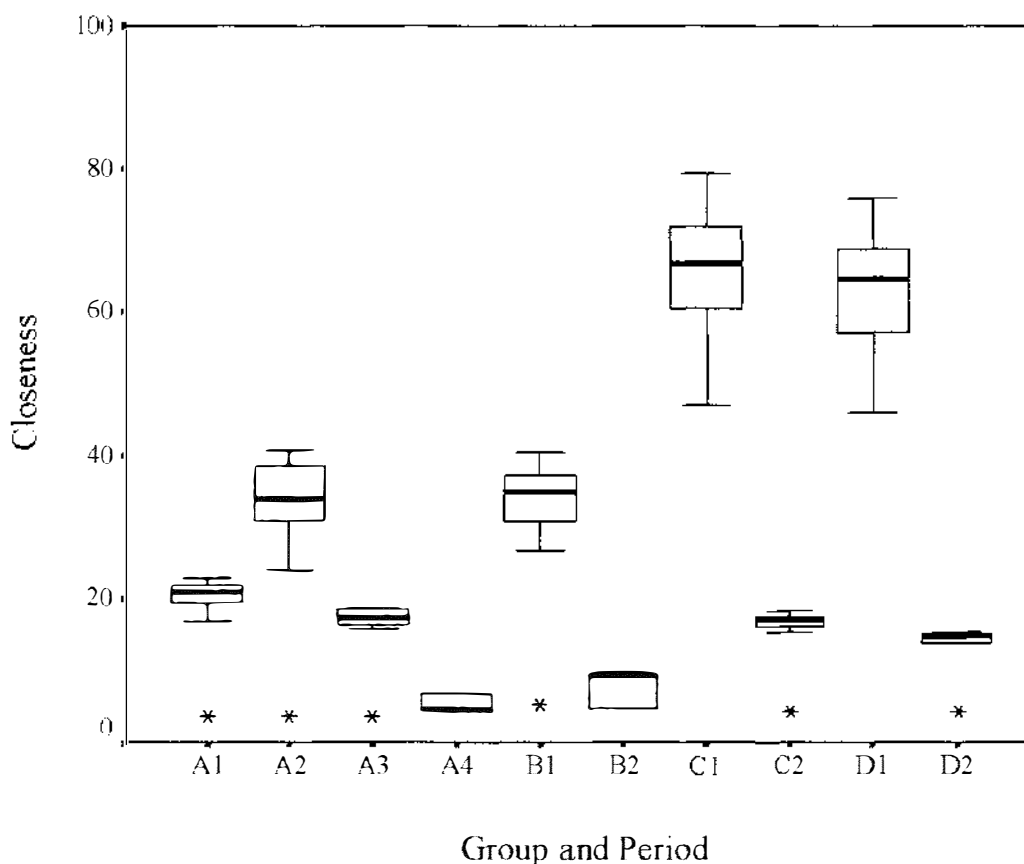


Figure 5.2. Observations on the behaviour of four groups (A, B, C and D) of communally housed captive brushtail possums. Each group was observed between 2 and 4 times. For Group A the environment of the pen was changed between periods A1 and A2, A2 and A3, and A3 and A4, and for Group B, 3 new members were added to the group between Periods B1 and B2.

Closeness centralisation index: In Group A, the index increased when the possum:den ratio increased (Period A1 to A2), decreased when the ratio decreased (Period A3 to A4), and again decreased after the possums were moved to another pen (Period A2 to A3). In Group B after they were moved to a new pen and 3 new members were introduced the index decreased. In Groups C and D, where the environment of the pens was left unchanged, the index decreased between periods.

Flow-betweenness: For Group A, this measure did not vary significantly between the first 3 periods but it was significantly lower in Period 4 when compared with the first three

periods ($p < 0.0001$ for each comparison). In Group B flow-betweenness decreased significantly between observation periods B1 and B2 ($p = 0.0001$). In the undisturbed Groups C and D, the mean flow-betweenness decreased significantly between periods ($p < 0.0001$ for each comparison).

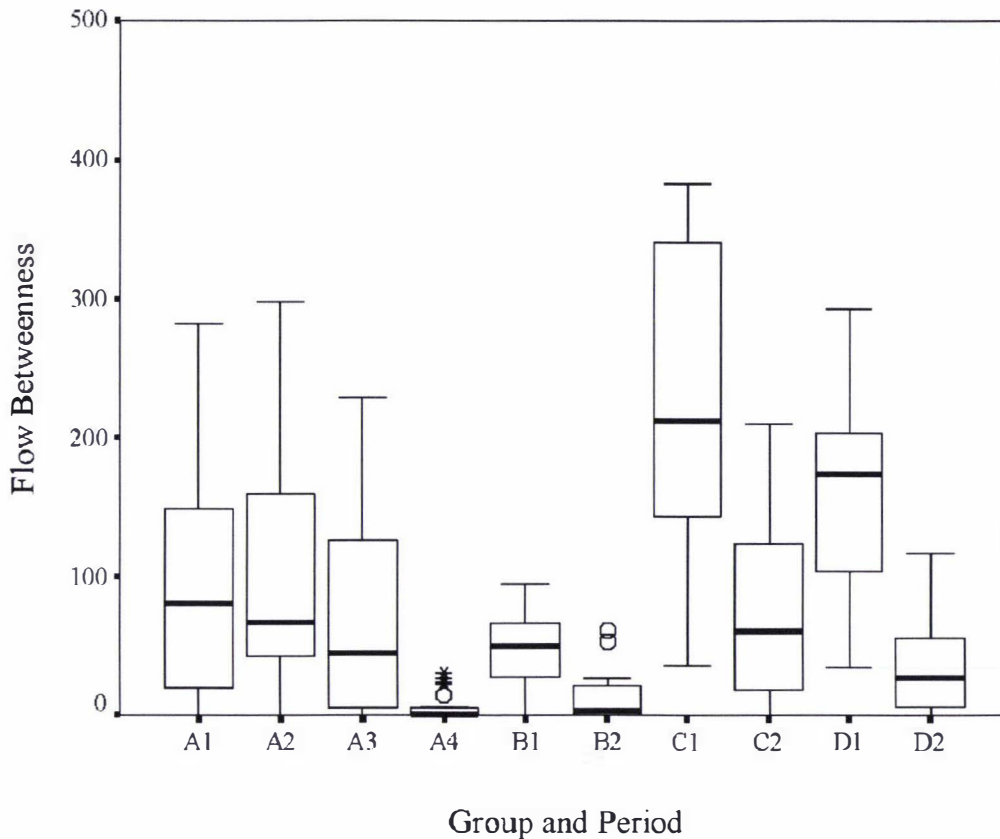


Figure 5.3. Observations on the behaviour of four groups (A, B, C and D) of communally housed captive brushtail possums. Each group was observed between 2 and 4 times. For Group A the environment of the pen was changed between periods A1 and A2, A2 and A3, and A3 and A4, and for Group B, 3 new members were added to the group between Periods B1 and B2.

Flow-betweenness centralisation index: For Group A, the index did not vary greatly when the first three periods were compared with each other but it was markedly higher in period A4. In Groups B, C and D, the index increased markedly between observation periods.

Individual's position in the network

The possums were ranked in each observation period according to their closeness and flow betweenness scores and their ranks were compared between successive periods (Table 5.3). For Group A, individual possums' ranks were highly correlated between periods but the degree of correlation declined progressively between successive periods. For Group B there was little correlation in possums' ranks between periods. New possums introduced before period B2 were not included in the ranking. In Group C there was a significant correlation in ranks between periods while there was little correlation in Group D.

No correlation was found between social position and body weight, nor between social position and changes in body weight (data not shown).

Table 5.3

Stability of the social rank of captive possums housed communally. The rank of each possums was determined separately for closeness and flow-betweenness.

Group	Periods		Correlation coefficient ^a	
	1 st	2 nd	Closeness	Flow-betweenness
A	A1	A2	0.91	0.85
	A2	A3	0.74	0.65
	A3	A4	0.59	0.69
	A1	A3	0.72	0.72
	A2	A4	0.66	0.64
	A1	A4	0.62	0.60
B	B1	B2	0.21	0.29
C	C1	C2	0.52	0.67
D	D1	D2	0.02	<0.1

^a Spearman rank order correlation coefficient (Cramer, 1997)

Tuberculosis transmission

Following experimental infection of 4 highly socially interactive possums in each of Groups C and D, 6 of 20 susceptible possums in Group C, and 12 of 19 susceptible

possums in Group D were found to have become infected with *M. bovis* when examined post mortem. In each group the possums that became infected had, significantly greater number of partners and shared dens more frequently during the transmission period than did those that remained free of infection (Table 5.4). A network diagram illustrating the interactions between possums for Group D in Period D2 is shown in Figure 5.4.

Table 5.4

Den use, number of partners and frequency of interactions for the 4 possums that were experimentally infected, those that became infected by transmission or remained free of infection in Groups C and D: data from the exposure period.

Group	Period	Disease Status ^a	Dens used		Partners		Interactions	
			mean	Sd	geometric mean	± 95% ci	geometric mean	± 95% ci
C	C2	Experimentally infected	2.0	0.4	2.3	2.0, 2.7	5.1	4.4, 5.8
		Naturally infected	2.2	0.9	3.2 *	2.9, 3.5	5.0 *	4.3, 5.8
		Free of infection	2.8	0.8	1.2 *	0.7, 1.8	2.0 *	1.1, 3.3
D	D2	Experimentally infected	2.5	0.4	1.8	1.5, 2.1	3.1	2.3, 4.1
		Naturally infected	3.2	0.6	2.0 *	1.5, 2.6	2.9 *	2.0, 4.0
		Free of infection	3.3	1.0	0.5 *	0.2, 0.9	0.7 *	0.2, 1.3

^a In each group den use, number of partners and interactions of the naturally infected possums and those that remained free of infection were compared using the *t* test (* $p < 0.05$).

The mean closeness and the mean flow-betweenness scores of the 4 possums experimentally infected in each group, those that became infected, and those that remained free of infection are shown in Table 5. At the time when disease transmission was occurring in both Groups C and D, there was a significant difference in closeness and flow-betweenness for the possums that became infected and those that remained free of infection. In analysing the effect of vaccination on the risk of disease transmission using

logistic regression, no effect of vaccination (OR = 1.1; (5% CI 0.4 – 3.1) was seen while controlling for closeness (OR= 0.97; 95% CI 0.94 – 1.00), flow-betweenness (OR = 1.01; 95% CI 1.003 – 1.021) and the groups to which possums belonged (OR = 0.13; 95% CI 0.04 – 0.41).

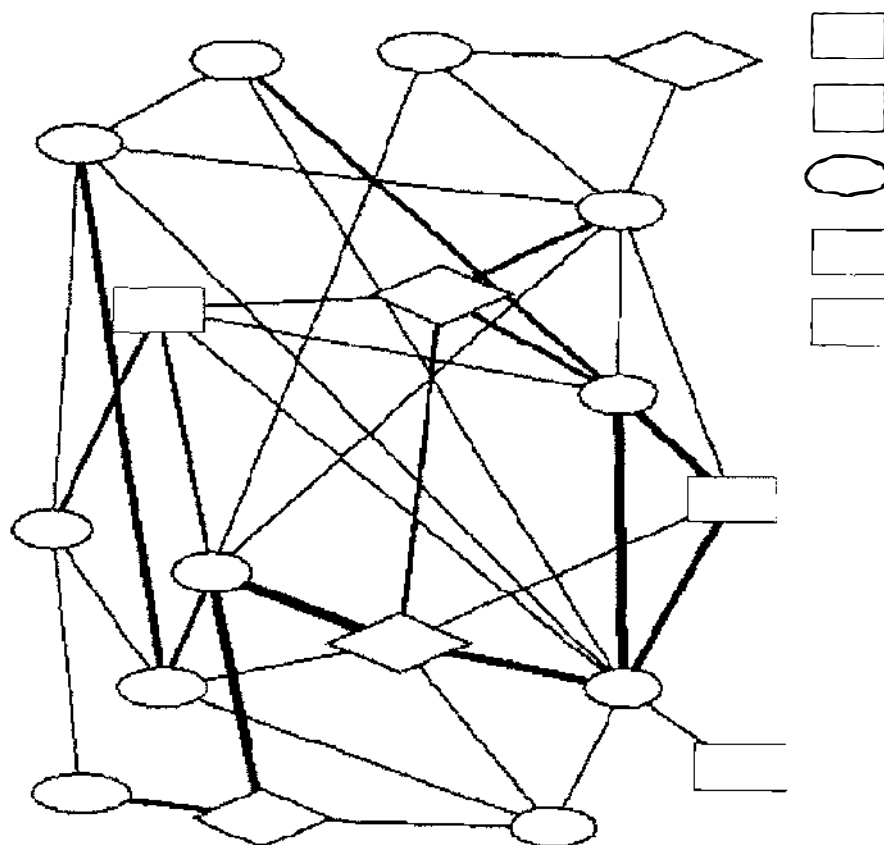


Figure 5.4. An example of a social network graph: Group D in Period D2, after the introduction of infection and during the period of exposure. The geometric forms represent possums: diamonds are experimentally infected possum, ovals are possums that acquired infection and rectangles are possums that remained free of infection. The five possums shown to the right of the network did not share a den with any other possums during the period. The width of the lines between possums denotes the frequency of the interaction (range 1 to 7.5).

Discussion

The structure and dynamics of the social organisation of groups of captive possums were analysed on the basis of den sharing behaviour, as an example of the use of SNA to

characterise the nature of disease transmission processes within a small population. SNA was a useful means of modelling social contacts between possums and it provided a comprehensive description of the social organisation. SNA was more comprehensive than analysis of partners or interactions, as it used more of the available data, combining the number of partners and the frequency of both direct and indirect interactions. The standardised measures enabled direct comparisons to be made between different groups and repeated observations on the same group. The analysis demonstrated that there were considerable differences between the behaviour of individuals in the groups, between groups and that there were changes over time.

Table 5.5

Mean closeness and flow-betweenness scores for the possums that were experimentally infected, those that became infected by transmission or remained free of infection in Groups C and D: data from the exposure period.

Group	Period	Disease Status ^a	Closeness		Flow-betweenness	
			mean	sd	mean	sd
C	C2	Experimentally infected	17.0	0.8	95.5	40.5
		Naturally infected	17.7 *	0.4	113.5 *	54.2
		Free of infection	13.4 *	6.1	50.3 *	62.8
D	D2	Experimentally infected	14.5	0.6	38.0	17.9
		Naturally infected	14.0 *	3.1	51.5 **	35.3
		Free of infection	8.7 *	5.5	8.1 **	15.2

^a In each group closeness and flow betweenness scores of the naturally infected possums and those that remained free of infection were compared using the *t* test (* $P < 0.05$, ** $P < 0.01$)

The most important and consistent influence on the social structure of groups was the length of time the group had been together in captivity. This was seen in all groups and was most marked immediately after the groups had been formed. Over time the social

distance between possums increased (that is, individual closeness scores decreased), the social network became more homogenous (decreased closeness centralisation index) and individuals became less differentiated from each other (individual flow betweenness scores decreased). However, with time the individuals in the group that were different to the majority were more prominent in the network (increased flow betweenness centralisation index). The measure that underwent the greatest change was closeness.

In each group during each observation period there was little variation in closeness scores between individuals, compared with the variation that occurred between observation periods. The range of values shown by individuals in a group in each observation period appeared to have been influenced by the behaviour of the group at that time, and was not an enduring characteristic of the individual. By contrast, individual flow-betweenness scores showed a wide range of values at each observation period and the range was less affected by the length of time the group had been in captivity. Flow-betweenness appeared to be a measure of an enduring characteristic of an individual. There were two exceptions to this. In Group B in both observation periods there was less variability in flow-betweenness scores than seen in other groups, and in Group A during the fourth observation period when many possums did not share a den.

Across all the possums studied, and within each group, there was great diversity in the way the available dens were used. However, individual possums were consistent in their behaviour. Some had a strong preference for particular dens and would use them repeatedly, while others used a variety of different dens. No possum used the same den more than 50% of the time and some possums changed dens daily. None of the groups, as a whole, used all the available dens, but showed preference for certain dens. There were no identifiable distinctive characteristics of the preferred dens.

In each group there were some possums that never, or rarely, used dens. In 8 of the 10 observation periods there were some possums that did not share a den with another possum and so were socially isolated. The possums isolated during one period were not always the same as those isolated during subsequent periods, but some individuals were isolated during more than one period. As the length of time that each group had been together increased, the number of possums that were isolated increased and in Group A in Period A4, the majority of possums were isolated. The number of isolated possums was affected

by changes to their environment. A decrease in the number of available dens increased the number of isolated possums and so did moving them to a new enclosure. This ran contrary to the effect of time.

Between groups the stability of the social organisation varied widely. In some groups there was a high correlation between observation periods and in others there was no correlation at all. In the group that had been together for the longest time, 8 months, there was an initial high correlation but it decreased over time. Among the 3 groups that were first observed after a month in captivity one showed a stable social organisation, but in the other 2 there was little stability. It is not possible therefore to reliably predict the social position of most possums at any one time based on past observations. However, there was a degree of stability in the members at the top of the social order. It is not clear if the instability in Group B was the result of the introduction of new members to the group or that the structure of the group was inherently unstable. The new members ranked low in the social structure as judged from their low closeness and flow-betweenness score.

The social network based on den sharing was a good model for describing the risk of disease transmission within groups. The social position of a possum had significant bearing on the risk of becoming infected, as those that became infected had significantly higher closeness and flow betweenness scores. That is, the infected possums were more central and prominent, than those that remained free of infection. As a result of experimentally infecting highly socially active possums in each group we achieved a high level of transmission of infection to in-contact possums. The rate of transmission was substantially higher than in previous studies where the possums that were experimentally infected had been randomly selected. There were different rates of transmission of infection to the in-contact possums in each of the groups with 30% and 63% becoming infected. However there were only minor differences in the measures of social behaviour for the groups overall and none clearly stood out as an explanation for the differences.

As shown in the present study, social position had a significant effect on the risk of transmission. In future studies of disease transmission within groups, social behaviour must be controlled, either in the design of the study or in the analysis of the data. Control for social position in the design of studies would be preferable, but unfortunately the social order of possums is not always stable over time. Therefore, social position will have to be

determined at the time of transmission and included as a confounding variable in the analysis of the data.

The analysis of den sharing did not fully describe the social behaviour of the possums nor did it account fully for the risk of infection. Observations on the denning behaviour were only conducted for 14 days at a time when the infected possums were expected to be excreting *M. bovis*. Den sharing was, however, a convenient and practical parameter to measure in a colony containing a large number of individuals and a meaningful parameter in the context of the disease of interest. Some transmission may have occurred during other social interactions but such interactions were difficult to measure. Disease transmission may also have resulted from sequential den use as *M. bovis* can survive in the environment for several days (Jackson et al., 1995b).

The risk of infection in the vaccinated and unvaccinated in-contact possums was compared using logistic regression treating social network measures as confounding variables, but no significant effect of vaccination was found. This finding is in sharp contrast to previous studies where vaccination provided significant protection (Aldwell et al., 1995, Buddle et al., 1997, Corner et al., 2001). One explanation for the lack of a vaccine effect may lie in the lack of sufficient statistical power, or the differences in social behaviour between the two groups.

The social organisation of captive possum groups was complex and involved many factors. The multidimensional nature of the social organisation described using SNA measures was easily appreciated when the social network was represented as a three-dimensional graph: with the third dimension being the frequency of interactions. The complex nature of these relationships was poorly represented by a simple hierarchy constructed through ranking possums on the basis of the number of different dens used, or the number of different partners or frequency of den sharing episodes. These latter analyses, however, provided additional information about the behaviour of captive possums.

The social structure of possum colonies can be described in a variety of ways and when different behaviours are used, different structures are described. The measures chosen will be relevant to and dependent on the use that will be made of the structure. Interactions that

may determine status of individuals for one purpose may be inappropriate for others. Studies on the social organisation and behaviour of captive possums usually focus on dominance relationships and mating behaviour (Day et al., 2000). The organisation is usually described in linear terms, such as hierarchies. However, they are more complex, with an individual's rank determined by factors other than simply the results of agonistic encounters (Biggins and Overstreet, 1978). When possums in male only groups were ranked on the outcomes of agonistic encounters, the position of the dominant individual was found to be stable (Biggins and Overstreet, 1978; MacGibbon, 1980, cited by Oldham, 1986). This was not the case when ranking possums on the number of partners and frequency of den sharing. Biggins and Overstreet (1978) found that new individuals in a colony occupied low positions in the hierarchy, which agrees with our findings.

The measures of social structure derived from SNA were highly relevant to the understanding and prediction of the patterns of disease transmission seen in the captive possums. Although simpler measures, such as the number of partners and the frequency of den sharing events, were significantly higher for the infected compared to the possums free of infection, the SNA-specific measures were more precise and were able to be compared across time and between groups. SNA was a useful tool for describing and analysing the social relationships quantitatively and investigating the relationship between an individual animal's role within its social group and the risk of disease transmission.

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Chapter 6

Natural transmission of *Mycobacterium bovis* in captive brush-tail possums (*Trichosurus vulpecula*) *

ABSTRACT:

Aims: To examine natural transmission of bovine tuberculosis (*Mycobacterium bovis* infection) in captive possums and to determine if it could be employed to challenge possums in vaccination studies.

Methods: Three experiments were conducted. In the first experiment pairs of possums were held in cages with one of the pair experimentally infected with *M. bovis*. Five of the 11 in-contact possums were vaccinated with BCG. In the second experiment, three susceptible possums were placed in a colony of 19 possums experimentally infected with *M. bovis*. In the third experiment, the 4 most socially active possums in each of two colonies (24 possums in one colony and 23 in the other) were experimentally infected with *M. bovis*, and 10 of the remaining possums in each colony were vaccinated with BCG.

Results: In the first experiment, transmission of infection occurred in only 1/11 pairs, but in the second experiment, none of the 3 in-contact possums became infected. In the third experiment, infection was transmitted to 5/20 in-contact possums in one colony and 12/19 in-contact possums in the second. The possums that became infected by natural transmission were significantly more socially interactive than those that remained free of infection ($p < 0.05$).

Conclusions: When susceptible and infected possums were randomly mixed, the rate of transmission of *M. bovis* was low, but when highly sociable possums were the source of infection the rate of transmission increased greatly. The risk of transmission was dependent on the close proximity of infected and in-contact susceptible possums and the frequency and duration of their social interactions. However, variation in the rate of transmission of infection make it unreliable for assessing vaccine efficacy.

INTRODUCTION

In New Zealand, bovine tuberculosis affects a range of domestic and wild animals, but it is the introduced brushtail possum (*Trichosurus vulpecula*) that is the major wildlife reservoir of *Mycobacterium bovis* (Morris and Pfeiffer, 1995). Infection is endemic in possum populations in over 30% of New Zealand (AHB, 2001). Infected possum populations are culled by trapping and poisoning programs to reduce the spread of infection to domestic livestock (Caley *et al.*, 1999). Culling of infected possum populations is a costly and unending process, for without continued control populations rapidly recover and the number of tuberculous possums with it. In the long term, new strategies for control of tuberculosis in possum populations will be required. Vaccination of possums against tuberculosis is a promising option (Buddle *et al.*, 2000).

In cross-sectional studies of wild populations, the majority of naturally infected wild possums had tuberculous lung lesions which contained large numbers of *M. bovis* (Cooke *et al.*, 1995). Approximately 75% of the wild tuberculous possums also had lesions in superficial lymph nodes and half of these lesions had developed into draining sinuses (Cooke *et al.*, 1995). The average length of clinical disease was estimated to be 2-6 months (Pfeiffer, 1994; Jackson, 1995), but the length of the pre-clinical phase remains unknown. The main route of transmission of infection between possums is thought to be the inhalation of infectious droplets excreted from the respiratory tract (Jackson, 1995).

There is strong circumstantial evidence to implicate tuberculous possums as the source of infection of domestic livestock (Caley *et al.*, 1999). Healthy possums are normally nocturnal and avoid the inquisitive behaviour of cattle and deer (Paterson and Morris, 1995). However, terminally ill possums have been observed to change their behaviour. They become active during daylight, appear dazed, weak and disoriented, and fail to respond when approached (Julian, 1981). Possums that behave abnormally are known to attract the attention of livestock, which investigate them by sniffing, licking or biting (Paterson and Morris, 1995; Sauter and Morris, 1995). Should livestock investigate a terminally ill tuberculous possum in this manner, infection could be transmitted by an infectious aerosol excreted by the possum.

Experimental infections have been established in possums using a variety of routes, including subcutaneous, intranasal, intramuscular and intratracheal inoculation (Bolliger

and Bolliger, 1948; Corner and Presidente, 1981; Julian, 1981; Pfeffer *et al.*, 1994). Intratracheal inoculation has been the method of choice for pathogenesis studies and for challenging possums in vaccination studies because it leads to a repeatable experimental disease with an assured level of exposure (Aldwell *et al.*, 1995; Corner *et al.*, 2001). However, all these procedures result in a disease characterised by a short period of clinical illness and the absence of palpable lesions in superficial lymph nodes.

Using the intratracheal challenge procedure, vaccination with *M. bovis* strain bacille Calmette-Guérin (BCG) has been shown to provide protection against disease, where protection was seen as a significant decrease in the severity of disease and less dissemination of infection following challenge (Aldwell *et al.*, 1995; Buddle *et al.*, 1997; Corner *et al.*, 2001). Under field conditions, vaccine efficacy was 69% (Corner *et al.*, 2000). However, studies of vaccine efficacy in captive possums using intratracheal challenge differ from conditions in the field, as all challenged possums develop progressive, rapidly fatal tuberculosis. The deficiency may lie in the challenge procedure, some aspect of the vaccination and challenge paradigm, or the possums may be immunocompromised due to the stresses associated with captivity (Buddle *et al.*, 1992).

The objective of the study reported in this paper was to determine if natural transmission between possums could be employed as a means of challenge in studies to determine the protection induced by vaccination. Natural transmission of tuberculosis between captive possums, by aerosol and contact, was first reported by Bolliger and Bolliger (1948), and later reported by others (Corner and Presidente, 1981; Buddle *et al.*, 1994). In these studies the possums were held in small cages. We undertook 3 experiments on natural transmission of tuberculosis in captive possums. In the first experiment pairs of possums were held in a small cage and 1 of the pair was experimentally infected. In the second experiment, 3 susceptible possums were housed with 19 experimentally infected possums as a single colony in a large pen. In the third experiment the 4 most socially active possums in each of two colonies, of 23 and 24 possums respectively, were experimentally infected and were the source of infection to the rest of the colony.

MATERIALS AND METHODS

Three experiments were undertaken and to a large extent the materials and methods were common to all three studies. In the first experiment, possums were caged in pairs and one of the pair was experimentally infected (Table 6.1). In the second, susceptible possums were housed with a colony of experimentally infected possums. In the third the most socially active possums in large colonies were experimentally infected and were the source of infection to the colony.

Table 6.1

Design of 3 experiments to study natural transmission of *Mycobacterium bovis* infection from experimentally infected to in-contact possums.

Variable	Experiment			
	1	2	3	
			Colony 1	Colony 2
No. infected (no. vaccinated)	11 (0)	19 (9)	4 (0)	4 (0)
No. of in-contact possums (no. vaccinated)	11 (5)	3 (0)	20 (10)	19 (10)
Selection of animals for treatment groups	Random	Random	Social behaviour	Social behaviour
Vaccination route	Intraduodenal	Conjunctival	Intranasal and conjunctival	Intranasal and conjunctival
Housing	2 possums per cage	Single colony	Single colony	Single colony

Experiment 1

Experimental Design: Eleven possums were housed individually and experimentally infected by intratracheal inoculation. Three and half weeks after infection a susceptible possum was placed in the cage with each experimentally infected possum. The experimentally infected possums were euthanased 3½ weeks after the introduction of the in-contact possums. The in-contact possums were euthanased 21 weeks after the commencement of the exposure period.

Possums: Eleven adult male and 11 adult female possums were captured in the Hutt Valley region of the North Island, and shown to be free of tuberculosis using the lymphocyte proliferation assay (LPA). The possums were housed in cages with a floor area of 90 cm x 68 cm. Eleven of the possums (10 males and one female) were experimentally infected by intratracheal inoculation with approximately 100 colony forming units (cfu) of *M. bovis* in 0.2 ml of broth (Pfeffer *et al.*, 1994). Three weeks after inoculation the remaining possums (10 females and one male) were housed with the experimentally infected possums so that each cage contained one inoculated possum and one in-contact possum, with a male and female combination in each pair. Five of the in-contact control possums had been vaccinated intraduodenally with 10^7 cfu of BCG 7 weeks prior to the start of sharing a cage with an experimentally infected possum. For intraduodenal inoculation, the duodenum was exposed following laparotomy and the BCG was injected through the wall of the duodenum using a 26 gauge needle (Buddle *et al.*, 1997).

Lymphocyte Proliferation Assay: The immunological response of possums to vaccination, experimental infection and exposure to infected possums was monitored using the lymphocyte proliferation assay (LPA) as described by Cooke *et al.* (1999). The bovine stimulation index (bovine SI) was calculated by dividing the mean counts per minute (cpm) for the triplicate lymphocyte cultures with bovine purified protein derivative (PPD) by the mean cpm for cells cultured with medium only.

Necropsy: The experimentally infected possums were killed 7 weeks after challenge and subjected to extensive post mortem examination. The presence and type of lesion was noted and confirmed as tuberculous by histopathological examination of haematoxylin and eosin (HE) and Ziehl-Neelsen (ZN) stains of tissue sections. Samples of lung and spleen were collected for bacterial culture. Surviving in-contact possums were euthanased and necropsied at the termination of the study, 21 weeks after the commencement of the period of exposure. Samples of lung, liver and spleen were collected for histological examination and samples from a pool of lymph nodes (bronchial, deep axillary and inguinal), lung and spleen were collected for bacteriology. Tissue samples were examined by standard techniques for the primary isolation of *M. bovis* (Comer *et al.*, 1995).

Experiment 2

Experimental Design: Three susceptible possums were housed communally in a pen with 19 experimentally infected possums. The three susceptible possums were introduced on the day that the 19 were experimentally infected with *M. bovis*. The experimentally infected possums died or were euthanased 8 weeks after infection and the in-contact possums were euthanased 20 weeks after the start of the exposure period.

Possums: Adult male possums were captured in the Tararua Ranges east of Palmerston North, in the North Island, an area free of possum tuberculosis. The possums were housed in a large outdoor pens with a floor area of approximately 200 m². Within the pen was a roofed shelter, enclosed on three sides, and in which were hung hessian bags for the possums to use as dens.

A colony of 19 possums was established as part of BCG vaccination and challenge experiment (Corner, unpublished observations). Nine of the possums were vaccinated and all 19 were experimentally infected as described for Experiment 1. On the day of infection three healthy adult male possums were introduced to the colony. The immunological response of the possums was examined using the LPA. A positive response was set as a bovine SI ≥ 6 , that is, equal to the mean of the pre-vaccination and pre-exposure assays plus two standard deviations above the mean.

Clinical Observations: The infected possums and the three in-contact possums were bled and weighed on the day of infection, and 4 weeks after infection. The in-contact possums were weighed at 7 weeks, and bled and weighed at 21 and 23 weeks. The den sharing of the three in-contact possum was observed for 7 days commencing 5 weeks after their introduction to the colony.

Necropsy: All surviving experimentally infected possums were euthanased 8 weeks after infection and the three in-contact possum at 24 weeks. All possums were subjected to a detailed necropsy. Body weight, the distribution of macroscopic lesions, and lung weight of each possum was recorded. Infection in the experimentally infected possums was confirmed by bacteriological examination of lung lesions. The disease status of the 3 in-contact possums was determined by bacteriological examination of a pool of tissues that

consisted of the mandibular, parotid, superficial axillary, deep axillary, and inguinal lymph nodes, spleen and lung.

Experiment 3

Experimental Design: Two groups of possums were housed separately in large outdoor pens. In order to ensure a high risk of exposure of the in-contact possums to the experimentally infected possums, the 4 most socially active possums in each pen were identified and experimentally infected.

Possums: Adult male possums were captured in the Tararua Ranges east of Palmerston North, in the North Island, an area free of possum tuberculosis. Two independent colonies, one of 24 and the other of 23 adult male possums, were established. The possums were housed as described for Experiment 2.

Determination of social structure: The social structure was described using social network analysis (SNA; Wasserman and Faust, 1994). The social network was determined by examining the interactions between individuals in the colony. The interaction of significance in the transmission of tuberculosis was the sharing of the enclosed airspace in the den, where the possums slept during the day. The social structure of the colony was determined by analyzing the den sharing behaviour, the number of other possums an individual interacted with and the frequency of interactions. A detailed description of the methodology will be published elsewhere.

Social network analysis describes the social position of each individual possum using both direct and indirect contacts, and provides structural models for analysing social relationships. In this study, two characteristics of the social network, closeness centrality and flow betweenness centrality are used. The concepts of closeness and flow betweenness are derived from graphic theory where social networks are represented as graphs, with animals as vertices and interactions the lines joining possums (Borgatti, 1995).

Centrality is a structural attribute of each individual in a network and is a measure of their importance or prominence in the network (DeGenne and Forsé, 1999). It is a measure of the extent to which the social network revolves around each possum. A centralisation index, which summarises the scores of all the individuals in the network, is a measure of

the heterogeneity of the network. The index allows comparisons of repeated observations on the same network.

Closeness is the sum of the distances of a given possum to all other possums in the network along the shortest path between them (Bell *et al.*, 1999). Closeness is calculated by summing the number of lines joining each possum to every other possum, using both direct and indirect interactions. The higher the closeness score the more central is the possum. As the closeness centralisation index increases, the network becomes more heterogeneous. It is maximum when one individual is central (maximum heterogeneity), and minimum when all are equally close.

If social interactions are visualised as establishing a communications network, then flow betweenness is a measure of the number of paths that pass through a possum, along the shortest paths between all other possums (Freeman *et al.*, 1991). It could be envisaged that the flow of communications between two indirectly linked possums is regulated by a possum that lies on the shortest path between them. The prominence or importance of each possum in the network is the number of indirect connections passing through it. The higher the flow betweenness score, the more prominent and influential is the possum. The flow betweenness centralisation index is minimum (0) when all individuals have exactly the same flow betweenness scores and maximum (1) when one individual lies between all others.

The social structure of each colony was determined twice, each for a period of 14 days. The second observation period was 3 months after the first and commenced 25 days after *M. bovis* infection was introduced into the colonies. After the first period, the possums in each colony were ranked on the frequency of den sharing and the number of different individuals they shared a den with. They were allocated to one of 3 treatment groups based on this ranking. In each pen 4 possums were selected for experimental infection. The subset was selected such that no other combination of 4 possums interacted with more possums or on more occasions. In order that the vaccinated and unvaccinated groups would have an equal risk of exposure, the remaining possums in each pen were ranked on social position and allocated alternatively to either the vaccinated group or the unvaccinated control group, such that the mean social ranking of each group was similar.

Vaccination and Experimental infection: At 4 weeks prior to the introduction of infection into the colony, possums in the vaccination groups were vaccinated and then held in isolation for 2 weeks. Possums were vaccinated by instilling into the conjunctival sac approximately 5×10^5 cfu of BCG and administering 200 μ l of the same BCG suspension as an aerosol directed at the external nares. The vaccinated possums were held isolated from the remainder of the colony for 14 days after vaccination. Experimental infections were conducted as described for Experiment 1. Infection was allowed to progress for 8-10 weeks, when the possums were euthanased.

Clinical Observations: The immunological response of possums to vaccination, experimental infection and exposure to infected possums was monitored using the LPA as described above. A positive LPA response was set as a bovine SI ≥ 6 , that is, equal to the mean bovine SI for the pre-vaccination and pre-exposure assays plus two standard deviations above the mean. All the possums were examined visually each day. They were weighed and bled 4 weeks before the commencement of the exposure period, that is, on the day vaccinations were performed, on the day infection was introduced into the colony (4 weeks after vaccination), and in Weeks 8, 12 and 16.

Necropsy: The experimentally infected possums were euthanased between Weeks 8 and 10 when they were terminally ill. The surviving in-contact possums were euthanased at Week 20. All possums were subjected to a detailed *post mortem* examination. At necropsy they were weighed and the presence of macroscopic lesions recorded. Tissues were collected for bacteriological and histology examination.

Where macroscopic lesions were detected, a representative sample was collected for bacteriological confirmation of infection. Where no macroscopic lesions were present a pool of tissues were collected for bacteriological examination. The pool consisted of lung, spleen, liver and the following lymph nodes: parotid, mandibular, retropharyngeal, deep and superficial axillary.

The following tissues were collected for histological examination: each lung lobe, spleen, liver, kidneys and adrenal glands. The following lymph nodes were also collected: parotid, mandibular, retropharyngeal, deep and superficial axillary, caudal cervical, inguinal, iliac and tracheo-bronchial, hepatic and mesenteric.

Data analysis: Descriptive statistics and the *t* test were performed in SPSS (version 9.0, www.spss.com.) and social network analysis was performed with the SNA software UCINET for Windows version 5.1.1.1 (Analytic Technologies, Harvard, Massachusetts, USA). The distribution of macroscopic and microscopic lesions were compared to that reported in wild naturally-infected possums (Jackson, 1995) using Spearman's rank order correlation (Cramer, 1998; Vose, 2000).

RESULTS

Experiment 1

Of the experimentally infected possums, one possum died 5 weeks after inoculation and the remaining possums were euthanased 6½ weeks after infection. All of the experimentally infected possums had extensive lung lesions. The median bacterial count in the lung lesions was \log_{10} 6.58 / g of tissue (range 5.28 to 7.42). Only one in-contact possum developed tuberculosis. It had been vaccinated with BCG and was euthanased *in extremis* 14 weeks after first sharing a cage with an infected possum. The infected in-contact possum had lost 0.5 kg (initial body weight 2.7 kg). The primary tuberculous lesion was in the lungs and there were miliary lesions in the liver and spleen. The other in-contact possums were in good condition at the time of necropsy, 20 weeks after first being caged with the experimentally infected possums, and no tuberculous lesions were observed. No *M. bovis* was isolated from the lungs, spleen and pool of lymph nodes of these in-contact possums.

In the vaccinated possums, the LPA response to bovine PPD remained elevated between 7-17 weeks after vaccination with a mean bovine SI of 20.6 (\pm 10.0), 15.7 (\pm 11.2) and 12.7 (\pm 8.4) at 7, 12 and 17 weeks after vaccination, respectively. These possums were placed with the experimentally infected possums 7 weeks after vaccination. In contrast, the LPA responses in the non-vaccinated in-contact possums remained low throughout the same period, with a mean bovine SI ranging from 1.3 (\pm 0.6) to 3.4 (\pm 2.5).

Experiment 2

Necropsy: Surviving possums were euthanased 7 weeks after experimental infection. In Week 6, two possums died and in Week 7, three possums died. All experimentally infected

possums had extensive lung lesions and *M. bovis* was isolated from all lung specimens. *M. bovis* was also isolated from the spleens of 7 vaccinated possum and 8 unvaccinated control possums.

The 3 possums in the in-contact group remained healthy and gained weight throughout the study. They were euthanased at 20 weeks after the start of the exposure period. At necropsy, there were no macroscopic signs of tuberculosis and bacteriological examination of tissues was negative for *M. bovis*.

Lymphocyte Proliferation Assay: The results of the LPA for the experimentally infected possums and for the three in-contact possums are shown in Table 6.2. At the time of challenge, 3 vaccinated possum had a positive bovine SI (≥ 6) but only one control possums was positive. By 4 weeks, all experimentally infected possums had a positive bovine SI.

In the in-contact group, one possum had a positive bovine SI at the commencement of the exposure period. At 4 weeks all 3 were positive and all the responses were approximately equal to, or greater, than the mean of both the experimentally infected groups. By 21 weeks the responses had decreased to insignificant levels and at 23 weeks one had a positive bovine SI.

Social behaviour: During the period of observation the 3 in-contact possums were seen to share dens with 7 other possums. Two of the in-contact possums shared a den with another possum on 2 separate occasions and the third shared dens on 2 occasions with another possum and on one occasion with 2 possums. Two of the possums sharing with the in-contact group were from the vaccinated group.

Experiment 3

Clinical signs: The experimentally infected possums became terminally ill and were euthanased between 8-10 weeks after infection. Five in-contact possums died from tuberculosis in Weeks 20-21, a vaccinated possum in Colony 1, and 3 unvaccinated and one vaccinated possum in Colony 2. Only 2 possums developed palpable lesions. One possum in the unvaccinated group in Colony 2 had a palpable lesion in the right inguinal lymph node (2 cm diameter) which was first detected at Week 16, and a vaccinated possum

in Colony 2 had palpable mass involving both parotid and both mandibular lymph nodes (3 cm diameter).

Necropsy: All of the experimentally infected possums had extensive lung lesions and tracheal swabs collected at necropsy from all the possums were positive for *M. bovis*. The study was terminated in Week 22 when all surviving in-contact possums were euthanased. In Colony 1, macroscopic lesions of tuberculosis were found in one of the 10 unvaccinated possums and 4 of the 10 vaccinated possums. In Colony 2, macroscopic lesions were found in 6 of the 9 unvaccinated possums and 6 of the 10 vaccinated possums (Table 6.3). *M. bovis* was isolated from all possums with macroscopic lesions but not from pooled tissues taken from possums without macroscopic lesions.

The distribution of lesions in the in-contact possums was very similar to those reported in wild, naturally infected possums (Table 6.3; for the macroscopic lesions the correlation coefficient was 0.66, and for the microscopic lesions, 0.64; Jackson, 1995). There were some significant differences. The captive possums showed less involvement of the superficial lymph nodes (superficial axillary, deep axillary and inguinal) and greater involvement of the lungs and the cranial mediastinal lymph nodes. In captive and wild possums there was a similar level of involvement of the abdominal organs and lymph nodes of the head and neck.

Experimentally infected possums – Four weeks after infection all these possums had a positive bovine SI. By 8 weeks only 2 were still alive, both were very debilitated due to advanced disease and they did not respond in the LPA.

Lymphocyte transformation assay: The number of possums in each group with a positive bovine SI are shown in Table 6.4.

Unvaccinated possums – After the introduction of infection into the pens the mean bovine SI of the unvaccinated possums rose slowly but few developed consistent positive responses. In Colony 1 only one possum had a persistent positive LPA response, from Week 12 to Week 20, and it had lesions of tuberculosis at necropsy. In Colony 2 the number of LPA positive possums increased to 7/9 at Week 16, and at necropsy of the seven positive, six had lesions.

Table 6.2

Immune response of possums experimentally infected with *Mycobacterium bovis*, and vaccinated and non-vaccinated in-contact possums. Experiment 2: Response to bovine purified protein derivative tuberculin (PPD) in the lymphocyte proliferation assay.

Weeks after experimental infection	Group								
	Control (n = 10)			Vaccinated (n = 9)			In-contact (n = 3)		
	Mean	sd	Positive *	Mean	sd	Positive	Possum 1	Possum 2	Possum 3
0	2.1	2.2	1	5.3	5.7	3	0.6	2.3	6.4
4	26.7	12.9	10	35.5	35	9	39.9	30.8	101.9
22							1.9	1.0	2.1
23							2.2	8.5	5.5

n = number of possums in the treatment group

*** A positive bovine stimulation index was ≥ 6.0**

Vaccinated possums – There was a similar pattern of response in each colony. By Week 4, 8/10 and 10/10 possums were LPA positive. The high LPA responses persisted until the end of the study.

Social behaviour: The social structure of the colonies was examined before and again after the introduction of infection. At each time point the colonies had similar closeness and flow betweenness scores, and centralisation indices. However, there was a significant decrease in closeness, flow betweenness and closeness centralisation indices between the first and second observation periods (data not shown). Between observation periods the possums had become less differentiated from each other and there was a decrease in the overall level of interaction. There was an increase in the flow betweenness centralisation indices, indicating that some individuals within the network were more prominent, although it was against a more homogeneous background.

Closeness and flow betweenness scores at the second observation period were compared to the disease status of the animals at necropsy (Table 6.5). In both colonies, those that acquired disease had significantly higher scores than those that remained free of infection.

Table 6.3

Distribution of macroscopic and microscopic lesions of bovine tuberculosis in possums experimentally infected by the intratracheal route (Experiment 3) and in naturally infected wild possums.

<i>Tissue</i>	<i>Captive possums</i>		<i>Wild, naturally infected*</i>	
	<i>n = 17</i>		<i>n = 73</i>	
	<i>Macroscopic</i>	<i>Microscopic</i>	<i>Macroscopic</i>	<i>Microscopic</i>
Left superficial axillary	0 [†]	13	41	60
Right superficial axillary	0	23	15	48
Left deep axillary	6	47	21	68
Right deep axillary	12	40	12	50
Left inguinal	6	40	19	52
Right inguinal	6	43	16	57
Left tonsil	0	21	0	22
Right tonsil	0	21	0	16
Left mandibular	6	40	1	18
Right mandibular	6	40	0	19
Left parotid	18	27	0	8
Right parotid	6	40	1	14
Left caudal cervical	0	43	1	15
Right caudal cervical	0	20	0	23
Left retropharyngeal	6	40	1	27
Right retropharyngeal	12	36	4	25
Left tracheo-bronchial	35	93	18	68
Right tracheo-bronchial	53	87	16	49
Mesenteric	24	80	26	61
Gastric	0	33	0	24
Hepatic	24	73	25	69
Liver	18	67	26	73
Spleen	18	47	21	49
Left kidney	6	40	23	35
Right kidney	29	33	34	48
Left adrenal	0	13	1	16
Right adrenal	0	13	0	12
Lung (any lobe)	94	94	75	85

* The distribution of macroscopic and microscopic in naturally infected wild possums as described by Jackson (1995).

[†] Percentage of tissues examined that had tuberculous lesions.

Table 6.4

Immune responses of possums in Experiment 3 - Response in the lymphocyte proliferation assay to bovine purified protein derivative tuberculin (PPD) in possums experimentally infected with *Mycobacterium bovis* and the vaccinated and unvaccinated in-contact possums.

Week *	Colony					
	1			2		
	Unvaccinated (1/10 [§])	Vaccinated (4/10)	Experimentally infected (4/4)	Unvaccinated (6/9)	Vaccinated (6/10)	Experimentally infected (4/4)
Positive [†]	Positive	Positive	Positive	Positive	Positive	
-4	1	0	1	0	0	0
0	0	2	0	0	1	0
4	0	8	4	1	10	4
8	1	8	0	1	5	0
12	2	10		3	10	
16	1	7		7	7	

* Vaccinations were done at Week -4 and experimental infections were done in Week 0.

[§] Number of possums with tuberculosis over the number in the group

[†] Positive - number of possums with a bovine stimulation index ≥ 6.0

DISCUSSION

In two of the three studies, tuberculosis was successfully transmitted from experimentally infected to susceptible in-contact possums. The rate of transmission varied considerably between experiments. In the first experiment only one of 11 in-contact possums, paired for 3½ weeks in a small cage with an experimentally infected possum, developed tuberculosis. In the second experiment, when three susceptible possums were housed in a colony containing 19 experimentally infected possums, no transmission occurred. A higher level of transmission was obtained in the third experiment where the experimentally infected possums were initially chosen because they ranked high in the social order of the colony. In one colony 63% of susceptible possums became infected and 26% in the second colony.

In the first experiment all the experimentally infected possums had extensive and progressive pulmonary lesions and there were very high bacterial counts from the lungs. However, only one in-contact possum developed tuberculosis. This possum had been

vaccinated. The in-contact possums that had been vaccinated all had elevated LPA responses to bovine PPD lasting for at least 17 weeks after vaccination. In a previous experiment, where possums were vaccinated in a similar manner, the response had decreased markedly by 9 weeks after vaccination (Buddle *et al.*, 1997). This persistent immune response to bovine PPD may have been in part due to exposure to live or dead *M. bovis* excreted by the experimentally infected possums, although no apparent infection was established in 4 of the 5 animals. It is clear from this study that mere close proximity of diseased and susceptible possums, that is, when confined in a small cage, is not sufficient to lead to a high rate of transmission.

Table 6.5

Measures of social interaction of possums: Mean closeness and flow betweenness, based on den sharing behaviour. The possums were grouped on their disease status at post mortem.

Colony	Disease Status	Closeness †		Flow betweenness	
		mean §	sd	mean	sd
1	Infected	17.7 *	0.4	113.5 *	54.2
	Free of infection	13.4 *	6.1	50.3 *	62.8
2	Infected	14.0 *	3.1	51.5 **	35.3
	Free of infection	8.7 *	5.5	8.1 **	15.2

† Closeness is based on graph theory and is the sum of the distances between each possum and all other possums in the network using the short path.

Flow betweenness is a measure of the number of paths that pass through each possum along the shortest paths between all other possums.

§ Within each colony, the scores for the infected possums and possums free of infection were compared using the t test with an adjustment for unequal variances (* $P < 0.05$ and ** $P < 0.01$)

In the second experiment, when three in-contact possums were held in a colony of 19 experimentally infected possums, there was no evidence of transmission of infection. There were opportunities for spread as during the exposure period the in-contact possums were seen to share dens with the diseased possums; although such den sharing was not

frequent. As in the first experiment, there was immunological evidence, from the LPA responses, that the in-contact possums were exposed to *M. bovis* or *M. bovis* antigens. Whatever the nature of the antigenic stimulation, it is clear that the experimentally infected possums were excreting *M. bovis* specific material by 4 weeks after infection. The responses may have been an anamnestic response to prior sensitisation from exposure to environmental mycobacteria. As in the first experiment mere close proximity, that is, sharing a den with a tuberculous possum was not sufficient to lead to the transmission of *M. bovis* infection and that other conditions must be met.

In the third experiment a greater rate of transmission was achieved with more of the in-contact possums developing tuberculosis. In this study the social structure of the colony was taken into account and the most socially active possums were chosen for the experimental infection group. This was done to ensure a high frequency of contact between the infected possums, excreting *M. bovis*, and the rest of the colony. The remaining possums in each colony were allocated to either the vaccinated or unvaccinated groups, such that the mean sociability scores of the groups in each colony were similar. This was done to ensure an equal risk of exposure of both vaccinated and unvaccinated possums to the experimentally infected possums. The selection of the possums for experimental infection proved successful with the rate of transmission being much higher than in previous experiments.

The number of possums that became infected in each colony was quite different. The difference in the rate of transmission between the two colonies cannot be explained by the selection procedures as the possums that were experimentally infected as in each colony were distinctly more socially active than the remaining possums. However, at the time of exposure there were differences in the social structure of the in-contact possum groups in the two colonies. In Colony 2, where the rate of transmission was higher, the possums were socially more homogeneous than in Colony 1 (closeness scores of 12 and 15 respectively) with fewer prominent individuals (flow betweenness scores of 36 and 74 respectively). The homogeneity and lack of prominent individuals in Colony 2 may have allowed more mixing of the in-contact and the infected possums.

Some tentative conclusions can be drawn from this study on the duration of the preclinical phase of tuberculosis in possums, which may be applicable to the disease in wild possums, but not obtainable from field studies. In Experiment 3, the LPA responses

first became significantly elevated at 8 weeks after the start of the exposure period in a non-vaccinated in-contact possum that became infected. The earliest any possum developed palpable lesions was between 12 and 16 weeks, a second possum developed palpable lesions between 16 and 20 weeks. The earliest a possum died was at 20 weeks. Therefore the minimum duration of the preclinical phase is estimated to be in the order of 8 to 20 weeks, when possums are held in captivity.

The distribution and frequency of lesions in the in-contact possums was similar to that seen in wild naturally infected possums. However, in the study with captive possums a greater proportion had lesions in lungs, thoracic lymph nodes and lymph nodes of the head and neck, but less involvement of the superficial body lymph nodes, compared with wild naturally infected possums. The distribution and frequency of lesions in the in-contact possums were indicative of aerosol transmission, with all but one possum having lung lesions. The high proportion of respiratory disease suggests that the pathogenesis in captive possums may differ from that in the wild. Dose rate, stresses associated with captivity, and the high possum density in the pens may have been factors contributing to the different pathogenesis. Also, the opportunity for transmission by other routes, for example agonistic encounters associated with mating and transmission via fomites, may have been absent from the pen environment.

Natural transmission of infection to susceptible in-contact possums may be a useful means of establishing infection when studying the pathogenesis of tuberculosis. The distribution of lesions was very similar to that seen in wild, naturally-infected possums. There was a wide range in the severity of lesions manifested in the in-contact possums, and in the distribution of lesions. Severity ranged from a possum with a single isolated parotid lymph node lesion, detected only at necropsy, to generalised disease with extensive lung lesions. Only one infected possum did not show lung lesions. Establishing infection by this procedure could be of particular use in studying the mechanism and the frequency of pseudovertical transmission. No other method of infecting possums published to date can be used for this purpose as each leads to fulminant infection and the rapid demise of the infected possums. Alternative methods are currently being investigated and appear promising.

Incubation times for naturally-infected wild possums have not been determined but the average survival times for infected possums, once they exhibit clinical signs, have been

estimated as 2-6 months. Survival times ranged from zero, where a possum died without showing clinical signs, to 36 months (Jackson, 1995). In the third experiment clinically detectable lesions were found in 2/17 (12%) possums at 16 and 20 weeks. In the wild, of possums with macroscopic lesions, 75% had clinically detectable lesions (Jackson, 1995). The low proportion of clinically detectable disease in the captive possum may indicate that disease develops rapidly in the pens.

The third experiment did not show the high level of vaccine efficacy that has been reported in other BCG vaccination studies using captive possums and in a study of a wild possum population (Corner *et al.*, 2000). Although an attempt was made to control for the confounding effect of social position, even when this was done no significant effect of vaccination was found. Variation in the rate of infection in the in-contact possums and the inability to effectively manage the variation in exposure make natural transmission an unreliable means of assessing the efficacy of vaccines.

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Chapter 7

Experimental Infection of Brushtail Possums (*Trichosurus vulpecula*) with *Mycobacterium bovis* by Conjunctival Instillation.*

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SUMMARY

In New Zealand, the brushtail possum (*Trichosurus vulpecula*) is the major wildlife reservoir of *Mycobacterium bovis*. Procedures for experimentally infecting possums are required to study the pathogenesis of the disease and to challenge possums in vaccine efficacy studies. Conjunctival instillation of a suspension of *M. bovis* was effective in producing experimental bovine tuberculosis in possums. The experimental disease progressed slowly with the development of palpable lesions in superficial lymph node, both characteristics of the disease in wild, naturally infected possums. At necropsy there was widespread distribution of macroscopic and microscopic lesions. The proportion of possums that became diseased, the rate of development and the severity of lesions, the severity of clinical signs all increased when the dose of *M. bovis* was increased. Of the three doses used, the medium dose (1000 to 2000 colony forming units) produced the disease with the most desired characteristics. As a procedure for exposing possums to infection with *M. bovis* the conjunctival route has advantages in that it is simple and safe to perform, and possums need only to be sedated for infection.

INTRODUCTION

In New Zealand, the introduced Australian brushtail possum (*Trichosurus vulpecula*) is the major wildlife reservoir of *M. bovis*. Infection is endemic in possum populations in many areas of both the North and the South Islands. Cattle are the natural host of *Mycobacterium bovis* and most mammalian species develop tuberculosis when infected with *M. bovis* (Thorne and Morris, 1983). Wild animal populations infected with *M. bovis* are a world-wide problem, as they can act as reservoirs of infection for domestic animals, other wild animal species and humans. Significant wild animal reservoirs have been identified in bison in Canada (Tessaro, 1986), cervids in the United States of America (Schmitt *et al.*, 1997), badgers in the United Kingdom and the Republic of Ireland (O'Reilly and Daborn, 1995; Hughes *et al.*, 1996) and African buffalo in South Africa (Keet *et al.*, 2000). Vaccination would make a very valuable contribution to tuberculosis control in wildlife, if it can be successfully applied.

Tuberculosis in naturally infected wild possums is primarily a respiratory disease, and commencing early in the disease course, it is common for possums to develop palpable lesions in superficial lymph nodes (Jackson, 1995). Experimental infection of possums has been used to study the pathogenesis of tuberculosis and to challenge possums in vaccination trials. Inoculation procedures that have been used include intraperitoneal inoculation (Bolliger and Bolliger, 1948), intramuscular inoculation (Corner and Presidente, 1980, 1981), and intratracheal inoculation (Buddle *et al.*, 1994; Aldwell *et al.*, 1995a; Cooke *et al.*, 1999). The rapidity of disease progression after experimental infection, the short survival times and the distribution of lesions were unlike that seen in naturally infected wild possums.

The best of the experimental infection procedures, intratracheal inoculation of a low dose of *M. bovis* (approximately 100 colony forming units), has been routinely employed in vaccination studies. It leads to a repeatable experimental disease with an assured level of exposure (Aldwell *et al.*, 1995a, 1995b; Buddle *et al.*, 1997; Corner *et al.*, 2001). However, infection by this route produces a rapidly fatal, fulminant pneumonia with possums rarely surviving for more than eight weeks. They do not develop the superficial lymph node lesions typically seen in natural cases.

Intratracheal challenge with *M. bovis* has been used to demonstrate that vaccination of captive possums with bacille Calmette-Guérin (BCG) induces protection, where protection was seen as less severe lesions, fewer *M. bovis* in lung and spleen lesions, and where vaccinates lost less body weight, than unvaccinated controls (Aldwell *et al.*, 1995; Buddle *et al.*, 1997; Corner *et al.*, 2001). However, in these studies both vaccinated and control possums developed lung lesions. The protection induced by vaccination may be better demonstrated if the experimental infection progressed more slowly, enabling the immune system to contain the infection at the initial site of infection. The conjunctival route of administration is effective for vaccinating possums with BCG (Corner, unpublished) and may be an alternative route for infecting possums. The objective of the current study was to investigate the characteristics of tuberculosis in possums infected by the conjunctival route and to compare the characteristics of the resulting disease with that following intratracheal inoculation.

MATERIALS AND METHODS

Experimental design

Three experiments were conducted in this study (Table 7.1). In Experiment 1, two groups of five possums were infected by conjunctival instillation of approximately 100 colony forming units (cfu) of *M. bovis* or 1000 cfu, and 12 possums were infected with approximately 150 cfu by the intra-tracheal route. In Experiment 2, three groups of 10 possum were infected with approximately 400 cfu, 2,000 cfu or 10,000 cfu, using the conjunctival route. In Experiment 3, 31 possums were infected by the intratracheal route with approximately 100 cfu.

The material and methods for Experiment 3 were described in detail in Corner *et al.* (2001). In that study the duration of protection induced by intranasal aerosol vaccination with bacille Calmette-Guérin was being investigated. Possums were vaccinated and then challenged by intratracheal inoculation at either two, six or 12 months after vaccination. At each time a similar number of unvaccinated controls were also infected. In each challenged group some possums died or were euthanased before then end of the study. Possums that survived until Week 7 after infection were then euthanased. The data from that study used in the current study was from the possums in the unvaccinated control groups that survived for at least seven weeks after infection.

Animals

Adult male possums were trapped in the North Island of New Zealand in an area free of possum tuberculosis. In Experiment 1 they weighed between 1.9 kg and 3.2 kg (mean 2.6 kg), in Experiment 2 between 1.8 kg and 3.9 kg (mean 2.8 kg) and in Experiment 3 between 2.0 kg and 3.3 kg (mean 2.7 kg). The communal housing of the possums in pens and handling of the possums were as previously described (Corner *et al.*, 2001). In each experiment, the possums were allocated to treatment groups from a list of the possums ranked on body weight. For clinical examination, weighing, bleeding and conjunctival infection, the possums were sedated with 100 mg to 150 mg of ketamine hydrochloride (Parnell Laboratories, East Tamaki, New Zealand) given by intramuscular injection. For intratracheal infection, they were sedated with ketamine and anaesthetised with Saffan (12 mg, Pet Elite, Lower Hutt, New Zealand). Possums *in extremis* or that survived to the end of the study were euthanased with an overdose of pentobarbitone.

Table 7.1**Conjunctival and intra-tracheal infection of possums with *Mycobacterium bovis*: Body weight changes.**

<i>Experiment</i>	<i>Route of inoculation</i>	<i>Dose *</i>	<i>Body weight at infection (kg) †</i>	<i>Proportional change in body weight ‡</i>
1	Intratracheal	100 cfu	2.79 (0.38)	- 0.33 (0.09)
	Conjunctival	Medium (1,000 cfu)	2.35 (0.19)	+ 0.07 (0.09)
	Conjunctival	Low (100 cfu)	2.20 (0.30)	+ 0.19 (0.09)
2	Conjunctival	High (10,000 cfu)	2.69 (0.35)	+0.29 (0.10)
	Conjunctival	Medium (2,000 cfu)	2.61 (0.37)	+ 0.04 (0.24)
	Conjunctival	Low (400 cfu)	2.74 (0.57)	+ 0.25 (0.19)
3	Intratracheal	100 cfu	2.69 (0.31)	-0.25 (0.09)

* cfu - colony forming units

† Mean (standard deviation)

‡ Change in body weight (kg) between infection and post mortem, divided by weight (kg) at infection; mean (standard deviation).

Experimental Infection

The *M. bovis* suspension was prepared using strain 83/6235, originally isolated from a naturally infected possum (Buddle *et al.*, 1994). The method of preparation of the challenge inoculum was as previously described (Aldwell *et al.*, 1995b).

Experiment 1. Possums were infected by placing into the conjunctival sac of each eye 50 µl of bacterial suspension. To accommodate the suspension the upper and lower eyelids were retracted and the eyeball was depressed. The eye was held in this position for 30 sec to 60 sec after the drop was delivered to allow absorption or drainage through the lachrymal duct. For the intratracheal inoculation the possums were anaesthetised and a plastic cannula (1.5 mm external diameter) was passed *per os* to below the bifurcation of the trachea (Aldwell *et al.*, 1995a). When in place 200 µl of the *M. bovis* suspension was instilled into the lungs and the cannula was flushed with an equal volume of sterile saline. The possums were then placed in lateral recumbency to recover.

Experiment 2. Possums were infected by placing into the conjunctival sac of each eye 10 µl of bacterial suspension. The 10 µl drop was readily accommodated without depressing the eyeball.

Necropsy

All possums were subjected to a detailed necropsy. At the examination, body and lung weights were recorded, as was the distribution of macroscopic caseous foci in the lungs, other organs and lymph nodes. Where macroscopic lung lesions were present a sample of the lesions (approximately 2 g) was collected for bacteriological examination. The ventral third of the spleen was collected for bacteriological examination from all possums. Where no macroscopic lesions were seen the following were collected for bacteriological examination: 1 g of each lung taken from the dorsal margin adjacent to the bifurcation of the tracheal, and a pool of lymph nodes that consisted of half of each of the following nodes: deep axillary, superficial axillary, mandibular, parotid and retropharyngeal.

Tissues were collected for histological examination. The following paired lymph nodes (LN) were collected: mandibular, parotid, retropharyngeal, caudal superficial cervical, deep axillary, superficial axillary, inguinal, iliac and tracheobronchial; and the following single or groups of lymph nodes: hepatic, gastric and mesenteric. Other tissues collected were the tonsils, liver, lung, spleen, kidneys and adrenal glands. For examination tissues were embedded in paraffin, sectioned at 3 µm, and stained with haematoxylin-eosin, and by the Ziehl-Neelsen method. Histological examination consisted of detection of tuberculous granulomas, characterised by the presence of acid-fast bacteria within the lesions. A tissue was classed as positive if it contained one or more tuberculous granulomas.

Bacteriology

The isolation of *M. bovis* from tissues samples was as described by Corner et al (1995) and their identification was as described by de Lisle and Havill (1985).

Lymphocyte Proliferation Assay (LPA)

Whole blood was collected for the lymphocyte proliferation assay (LPA) from each possum at the times shown in Table 7.2. Blood was collected from the jugular vein and preserved in heparin. The proliferative response of peripheral blood lymphocytes was

measured using the method described by Cooke *et al.* (1999). Briefly, 1 ml of blood was added to 50 ml of lysing buffer containing 0.17 M Tris and 0.16 M NH_4Cl , pH 7.2 and incubated at 37 °C for 10 min. The cell suspension was centrifuged at 350 g for 10 min, resuspended and washed twice in PBS. The cells were finally resuspended in RPMI 1640, containing 2% normal possum serum, 2 mM glutamine and antibiotics. To each well of a sterile cell culture microtitre plate was added 200 μl of cell suspension containing approximately 1×10^6 mononuclear cells / ml. Cells were cultured with bovine purified protein derivative (PPD; CSL Ltd, Melbourne, Australia, 60 $\mu\text{g}/\text{ml}$ final concentration) or Concanavalin A (Con A; Sigma, St Louis, MO, USA, 5 $\mu\text{g}/\text{ml}$ final concentration). For the unstimulated control wells, 50 μl of RPMI 1640 medium was added. Assay conditions, harvesting and β -scintillation counting were as described previously (Cooke *et al.*, 1999). The LPA response to bovine PPD was expressed as the bovine stimulation index (bovine SI) and was calculated by dividing the response to bovine PPD by the response to media alone. A positive LPA response was taken as a bovine SI ≥ 4 , that is, equal to the mean bovine SI before infection plus two standard deviations above the mean.

Statistical analysis

The distribution of macroscopic and microscopic lesions in different experiments were compared using Spearman's rank order correlation coefficient (Cramer, 1998; Vose, 2000).

RESULTS

Experiment 1

Intratracheal infection

Clinical observations and necropsy: Wasting, as a consequence of advanced tuberculosis, was seen in most possums and was first detected six weeks after infection. No palpable lesions were detected in any possum when they were examined four weeks after challenge. All possums in this group lost body weight between infection and necropsy (Table 7.1). Ten possums died and two were euthanased *in extremis*. One died in Week 6, five died and one was euthanased in Week 7, three died in Week 8 and one died and one was euthanased in Week 9. All had extensive macroscopic lesions in the lungs and

bronchial lymph nodes. Macroscopic lesions were found in a range of other tissues: spleen (6 possums), liver (4), hepatic lymph node (5), kidneys (5), adrenal glands (1), mesenteric lymph nodes (2), mandibular lymph node (1), and the wall of the caecum (1). *M. bovis* was isolated from the lungs of all possums.

Table 7.2

Conjunctival and intra-tracheal infection of possums with *Mycobacterium bovis*: immune response to bovine purified protein derivative in the lymphocyte proliferation assay.

Experiment	Route of Inoculation	Dose *	Weeks after infection						
			0	4	6	8	12	16	20
1	Intratracheal	150 cfu	0/12	12/12	nd †	2/5	§		
	Conjunctival	Medium - 1000	1/5	1/5	nd	3/5	2/5	0/5	2/5
	Conjunctival	Low - 100 cfu	0/5	0/5	nd	3/5	2/5	1/5	1/5
2	Conjunctival	High - 10000	0/10	nd	7/10	nd	§		
	Conjunctival	Medium - 2000	0/10	nd	5/10	nd	6/10	nd	§
	Conjunctival	Low - 400 cfu	0/10	nd	1/10	nd	1/9	nd	0/9

* cfu - colony forming units

† Number of possums with a positive bovine stimulation index (SI) over the number of possums in the group. A positive LPA response was a bovine SI ≥ 4

‡ nd - blood was not collected at these times

§ All the possum in the group had died or had been euthanased

Lymphocyte proliferation assay: At Week 4 all possums were LPA positive with a mean bovine SI of 69.8 (Table 7.2). By Week 8, of the five possums that were still alive, only two gave positive responses. The three possum negative on the LPA were debilitated and had very low responses to Con A.

Conjunctival infection

Clinical observations and necropsy: Palpable lesions were first detected in one possum of the high dose group (822) in Week 20, immediately prior to the group being euthanased. The lesions, 10 mm diameter swellings, were palpated in the left and the right inguinal

lymph nodes and *M. bovis* was isolated from the lesions. No lesions were detected in any of the remaining members of this group and all gained body weight between infection and necropsy (Table 7.1).

Lymphocyte proliferation assay: At each time point there were possums that had a positive LPA response, but there was no consistent pattern (Table 7.2). Possum 822, which had a palpable lesion was LPA positive only at Week 12.

Experiment 2

The high dose group was euthanased in Week 9 after three possums developed draining sinuses. In the medium dose group, Possum 893 died in Week 15 and the remainder of the group were euthanased in Week 16, after Possum 877 developed a draining sinus. The possums in these two group were euthanased because there was a possibility that the draining sinuses would act as an additional source of infection. In the low dose group Possum 792 died in Week 12 and the remainder were euthanased in Week 20.

High Dose Group

Clinical observations: By Week 6, five possums had swellings of 15 mm to 30 mm diameter in parotid LN, mandibular LN or both. By Week 9, these five retained the swellings and in four, the swelling had developed into a draining sinus. By this time two other possums had developed swellings. Between infection and necropsy all the possums increased or maintained their body weight (Table 7.1).

Lymphocyte proliferation assay: By Week 6, seven possums had a positive bovine SI (Table 7.2) and all had macroscopic lesions at necropsy at Week 9 (Table 7.3). The three possums with a negative bovine SI had no macroscopic lesions at necropsy but two had microscopic lesions.

Necropsy: The number and distribution of macroscopic and microscopic lesions is shown in Tables 7.3 and 7.4. Seven possums had macroscopic lesions, six had lung lesions, primarily affecting the caudal lobes, and two had lesions in the tracheobronchial LNs. Macroscopic lesions were seen in 14 extra-thoracic sites (Table 7.4), primarily affecting the lymph nodes of the head and neck, but also the abdominal organs. One possums (876) with lesions in 12 sites contributed disproportionately to the wide distribution of lesions.

Microscopic lesions were seen in nine possums (Table 7.3), with lesions in the lungs and tracheobronchial LNs of five. A wide distribution of microscopic lesions were seen but possum 876 contribute disproportionately to the distribution. Microscopic lesions were seen in 20 extra-thoracic sites (Table 7.4). *M. bovis* was isolated from all affected lungs and from three spleens with macroscopic lesions, and two spleens with no macroscopic lesions.

Table 7.3

Conjunctival infection of possums with *Mycobacterium bovis*: number of macroscopic and microscopic lesions. The low dose group was euthanased at 20 weeks after infection, the medium dose group at 16 weeks after infection and the high dose group at 9 weeks after infection.

<i>Dose</i>	<i>Macroscopic lesions</i>			<i>Microscopic lesions</i>		
	<i>N</i> *	<i>Median</i>	<i>Range</i>	<i>N</i>	<i>Median</i>	<i>Range</i>
High - 10000 cfu †	7	3	2 – 12	9	5	2 - 14
Medium - 2000 cfu	9	7	1 – 10	10	9	1 - 27
Low - 400 cfu	1	NA ‡	5 lesions	2	NA	1 & 11 lesions

* Number in each group of 10 possums that was affected.

† cfu - colony forming units

‡ NA - not applicable

Medium Dose Group

Clinical Findings: Five possums developed palpable swellings. By Week 6, four possums had 10 mm to 15 mm diameter swellings in parotid LN, mandibular LN or both. By Week 9, swellings were present in these four possums and one additional possum (893) had a 15mm swelling in the left parotid LN. By Week 12 Possum 893 no longer had a palpable lesion but was clinically ill and was losing weight. By Week 15 two of the five possums, 893 and 786, had died, two possums still had palpable swellings (727 and 782) and a third (877) had a 10mm sinus draining the left parotid LN.

Lymphocyte proliferation assay: Five possums were positive at Week 6 and six were positive at Week 12; all six had lesions at necropsy.

Necropsy findings: The number and distribution of macroscopic and microscopic lesions is shown in Tables 7.3 and 7.4. Nine possums had macroscopic lung lesions and eight also had lesions in other sites. The lung lesions involved between three and six lobes (median six lobes). Four possums had macroscopic lesions in the tracheobronchial LNs. Macroscopic lesions were seen in 17 extra-thoracic sites (Table 7.4).

Microscopic lesions were seen in all 10 possums (Table 7.3) with microscopic lesions in the lungs and tracheobronchial LNs of nine and in 26 extra-thoracic sites (Table 7.4). One possum, (731), where no macroscopic lesions were seen, had microscopic lesions in a mesenteric LN. *M. bovis* was isolated only from possums with macroscopic lung lesions.

Low Dose Group

Clinical findings: Only one possum, 792, developed clinical signs. It lost 750 g in body weight between Weeks 6 and 9, and died in Week 12. The remaining members of this group gained weight (Table 7.1).

Lymphocyte proliferation assay: Possum 792 developed a positive LPA at Week 6 and died with extensive lesions of tuberculosis in Week 12. Possum 5968 had a positive response at Week 12, was free of lesions at necropsy and was negative on bacteriological examination. Possum 787 was negative throughout the study but *M. bovis* was cultured from lung samples. The remaining possums in the group were negative throughout the study.

Necropsy findings: The number and distribution of macroscopic and microscopic lesions is shown in Tables 7.3 and 7.4. Possum 792 had macroscopic lesions in all six lung lobes, and lesions in both tracheobronchial LNs, kidneys, spleen and hepatic LN. *M. bovis* was isolated from lung and spleen. Microscopic lesions were seen in 11 sites in possum 792. Small numbers of *M. bovis* (50 cfu) were isolated from the lungs of Possum 787, and a microscopic lesion was seen in a gastric lymph node.

Table 7.4

Number and distribution of macroscopic and microscopic lesions in possum infected with *Mycobacterium bovis* by conjunctival inoculation (Experiment 2).

<i>Tissue</i>	<i>Dose *</i>					
	<i>High - 10000 cfu</i>		<i>Medium - 2000 cfu</i>		<i>Low - 400 cfu</i>	
	<i>Macroscopic</i>	<i>Microscopic</i>	<i>Macroscopic</i>	<i>Microscopic</i>	<i>Macroscopic</i>	<i>Microscopic</i>
Left superficial axillary	0 †	0	0	29	0	0
Right superficial axillary	0	10	10	13	0	0
Left deep axillary	0	10	0	38	0	0
Right deep axillary	0	20	10	38	0	0
Left inguinal	0	10	10	22	0	0
Right inguinal	0	10	0	25	0	0
Left tonsil	0	11	0	80	0	0
Right tonsil	0	11	0	43	0	0
Left mandibular	30	33	40	44	0	0
Right mandibular	20	33	10	38	0	0
Left parotid	20	20	50	56	0	0
Right parotid	40	40	10	14	0	0
Left caudal cervical	20	30	40	50	0	0
Right caudal cervical	20	40	0	38	0	0
Left retropharyngeal	20	33	30	71	0	0
Right retropharyngeal	10	44	20	71	0	0
Left tracheo-bronchial	20	50	40	89	10	13
Right tracheo-bronchial	20	33	30	90	10	13
Mesenteric	0	60	50	100	0	10
Gastric	10	20	10	38	0	11
Hepatic	30	50	20	63	10	10
Liver	10	20	10	60	0	10
Spleen	30	30	40	50	10	10
Left kidney	0	0	30	40	0	10
Right kidney	10	10	10	22	10	10
Left adrenal	10	0	0	40	0	10
Right adrenal	0	0	0	22	0	10
Lung (any lobe)	60	50	90	90	10	10

* There were 10 possums in each treatment group. cfu - colony forming units

† Percentage of tissues with lesions

Table 7.5

Number and distribution of macroscopic and microscopic lesions in captive possums experimentally infected with *Mycobacterium bovis* by intratracheal inoculation (Experiment 3) and naturally infected wild possums

<i>Tissue</i>	<i>Intratracheal infection</i>		<i>Naturally infected*</i>	
	<i>(N = 31) †</i>		<i>(N = 73)</i>	
	<i>Macroscopic</i>	<i>Microscopic</i>	<i>Macroscopic</i>	<i>Microscopic</i>
Left superficial axillary	0 ‡	18	41	60
Right superficial axillary	0	16	15	48
Left deep axillary	0	32	21	68
Right deep axillary	0	40	12	5
Left inguinal	0	32	19	52
Right inguinal	0	28	16	57
Left tonsil	0	9	0	22
Right tonsil	0	13	0	16
Left mandibular	0	10	1	18
Right mandibular	0	18	0	19
Left parotid	0	7	0	8
Right parotid	0	14	1	14
Left caudal cervical	0	17	1	15
Right caudal cervical	0	13	0	23
Left retropharyngeal	0	34	1	27
Right retropharyngeal	0	28	4	25
Left tracheo-bronchial	81	94	18	68
Right tracheo-bronchial	97	100	16	73
Mesenteric	3	71	26	61
Gastric	3	29	0	24
Hepatic	16	58	25	69
Liver	7	97	56	73
Spleen	10	43	21	49
Left kidney	16	23	23	35
Right kidney	13	27	34	48
Left adrenal	0	17	1	16
Right adrenal	0	0	0	12
Lung (any lobe)	100	100	75	85

* The distribution of macroscopic and microscopic in 73 naturally infected wild possums as described by Jackson (1995).

† N = number of possums examined

‡ Percentage of tissues with lesions

Experiment 3 and Naturally infected possums

The number and distribution of macroscopic and microscopic lesions in 31 possums infected by the intratracheal route are shown in Table 7.5. All possums had macroscopic lung lesions, with no lobes being preferentially affected, and all had lesions in at least one tracheobronchial LN. Macroscopic lesions were seen in 7 extra-thoracic sites and all were in the abdominal cavity (Table 7.5). Microscopic lesions were present in lungs and tracheobronchial LN in all possums, and in 25 other sites (Table 7.5). Following the lungs, the most frequently affected organs were the liver (97%), hepatic LN (58%) and mesenteric LN (71%).

Jackson (1995) described the distribution of macroscopic and microscopic lesions in 73 naturally infected wild possums (Table 7.5). The tissues most frequently found to contain macroscopic lesions were the lungs, liver and superficial LNs of the body (superficial and deep axillary LN, and the inguinal LNs) and with a low frequency of lesions in lymph nodes of the head and neck. Microscopic lesions were widely distributed throughout the body, principally in the thoracic cavity, abdominal cavity and the superficial LNs of the body.

Comparison of the distribution of lesions in experimentally infected and naturally infected possums.

The distribution of macroscopic and microscopic lesions in possums infected following conjunctival infection with the medium and high doses, or by the intratracheal route, were compared with that reported in 73 naturally infected wild possums (Table V; Jackson, 1995). As there was only one possum with lesions after low dose infection, no analysis was conducted. Overall there was poor correlation between the distributions of macroscopic lesions between the naturally infected and experimentally infected possums. The intratracheal group had the highest correlation (0.53), with the medium dose group (0.21) and high dose group (-0.10) showing no correlation. The correlation was higher when the distributions of microscopic lesions were compared. The intratracheal and medium dose groups were similar (0.76 and 0.72 respectively) but the high dose group showed poor correlation (0.21).

DISCUSSION

Tuberculosis in captive possums was induced by instilling a suspension of *M. bovis* into the conjunctival sac. The proportion of possums that became diseased, the rate of development and severity of lesions, the severity of clinical signs, and distribution of macroscopic and microscopic lesions, were all dependent on the dose of *M. bovis*. No visible lesions developed on the conjunctivae of infected possums.

Increasing dose rate produced an increase in the severity of the infection and the time course of disease was shortened. The medium dose group in Experiment 2 led to the development of tuberculosis in nine out of 10 possums. The disease in these possums progressed slowly, compared with those infected by the intratracheal route, and was characterised by the development of palpable lesions in superficial lymph nodes in five possums and a draining sinus in one. Lower and higher dose rates gave different results. A five fold lower dose (400 cfu) led to disease in only two of 10 possums and a five-fold higher dose (10,000 cfu) led to a more rapid disease process.

Conjunctival infection produced disease with the characteristics that were desired: a slowly developing disease, with superficial lymph node lesions and widespread evidence of microscopic infection. The disease resulting from conjunctival infection had a less dramatic effect on the general health of the possums than did intratracheal infection. Even with the highest dose rate the diseased possums did not become debilitated and the majority gained body weight. The highest dose led to the rapid development of superficial lymph node lesions and the development of draining sinuses. That group was euthanased in the ninth week after infection because there was a high risk of transmission of disease from open draining sinuses. By about eight weeks after intratracheal infection the possums had either died or were severely debilitated. The disease in these possums was characterised by extensive lung lesions.

The optimum dose for conjunctival infection was between 1000 cfu and 2000 cfu. This dose resulted in disease with a course that reflected tuberculosis as seen in naturally infected possums. The possums had slowly progressing infection and in the second experiment half of the group receiving this dose developed clinically detectable swellings by nine weeks and 90% had pulmonary disease. At 15 weeks one possum died from advanced pulmonary disease and another had developed a draining sinus. Apart from the

possum that died, the possums remained in good health and half had increased in body weight at the end of the experiment. As with the high dose group, this group was euthanased because of concerns regarding horizontal transmission from a draining sinus.

The possums infected by the conjunctival route were euthanased at fixed times after infection and there was a wide range in the extent to which the disease had progressed. This is similar to the variable rate of disease progression seen in naturally infected wild possums (Jackson, 1997). Other than swellings in superficial lymph nodes, few possums in the conjunctival infection groups showed signs of ill health. The possums in the medium dose group especially would have survived for many more weeks. It could be expected that with more time the conjunctival group would have become more like the naturally infected possums in the distribution and frequency of macroscopic and microscopic lesions.

The conjunctival route has advantages over other routes for the experimental infection in possums. It is a simple procedure, requiring only sedation of the subject, and is non-invasive. Infection by this procedure required a dose of *M. bovis* 20 to 40 times higher than used to produce infection by the intratracheal route. Inoculation leads to slowly developing disease, with a long preclinical phase. Using this challenge procedure it may be possible to demonstrate more effectively the protective potential of vaccines, especially for demonstrating subtle differences between vaccines not discernible with more robust challenge procedures. Vaccine studies using this route of infection in captive possums may be substituted for expensive field studies.

The survival time after conjunctival infection with the medium dose mimicked that seen in naturally infected wild possums. Naturally infected wild possums survived for 2 to 6 months after clinical disease was first detected (Pfeiffer, 1994; Jackson, 1995). In the medium dose group, four of the five possums that developed clinical disease were still alive nine weeks after the lesions were detected and were still in good health. They might have survived another 4 to 8 weeks based on the extent of their lung lesions at necropsy.

There was a possibility that horizontal transmission of *M. bovis* infection occurred between possums in two of the conjunctival inoculation groups. In the low dose group, *M. bovis* was isolated from the lungs of a possum that had no lesions at necropsy. This is suggestive of early aerosol infection and the source was probably a member of the group that had extensive lung lesions and died during Week 12. In the medium dose group, two

possums, both of which had maintained low LPA responses throughout the study, had small thorax and abdominal cavity lesions at Week 16. Six members of this group had extensive lung lesions and could have been the source of an infectious aerosol. One possum in this group developed a draining sinus at Week 15, which was probably too late in the study to have been the source of the secondary spread.

Direct infection of the lungs appears to have occurred as an unexpected side effect of the conjunctival inoculation procedure in one possum. A possum in the low dose group in Experiment 2 died in the twelfth week with predominantly pulmonary tuberculosis. Pulmonary infection probably arose after the inoculum drained through lachrymal duct and was aerosolised in the posterior nasal cavity. The risk of this happening again could be minimised by reducing the volume of the inoculum.

The duration of pre-clinical infection in wild possums is not known because the point of exposure cannot be ascertained. The current studies have given some insights into the duration of this phase of infection. The majority of conjunctivally infected possums developed palpable lesions by six to nine weeks after inoculation, and in one it was 20 weeks before such a sign was seen. It is probable that in the wild the time from infection to the development of clinical signs will vary with the number of bacteria in the infecting dose and the route of infection but is unlikely to be less than 6 to 9 weeks.

In the studies on experimental infection by Aldwell *et al.* (1995a, 1995b), Buddle *et al.* (1994) and Corner *et al.* (2001) the possums were all caught in the wild and allowed to adjust to captivity before experimentation commenced. The rapid progression of experimental disease may have been due to the combination of the experimental infection procedures and animals not fully adapted to confinement. Possums captured from the wild experience stress due to a variety of causes including an unusual diet, close human contact and close confinement (Presidente and Correa, 1981; Buddle *et al.*, 1992). These stresses would increase their susceptibility to experimental infection. When possums were captured, experimentally infected by the intratracheal route and then released back into the wild, the median survival time was 11 weeks, with one possum surviving until 20 weeks after infection (Corner unpublished observation).

A successful experimental infection procedure does not, of necessity, have to reflect the natural route of infection to be useful in pathogenesis studies or for challenging animals in

vaccination studies. The appeal of the conjunctival infection procedure is its simplicity and the nature of the resulting disease. In this respect it may have application in other species, such as the badger or deer. It is easier and less hazardous to perform than inoculation into the tonsillar crypt, the current procedure used successfully for pathogenesis and vaccine studies in deer (Mackintosh *et al.*, 1995; Griffin *et al.*, 1999; Palmer *et al.*, 1999). In badgers experimental disease has been established by intradermal inoculation, in calves by the intranasal inoculation of a suspension of *M. bovis* containing between 10^4 and 10^6 bacteria (Neill *et al.*, 1988, 1989). In all these species tuberculosis is primarily a respiratory disease.

There is compelling pathological and field data that implicates aerosol transmission as the natural route of transmission between adult possums in the wild (Jackson *et al.*, 1995). The results of our studies do not change this understanding. All the same, conjunctival infection provides an additional, useful procedure for studying the pathogenesis of tuberculosis in possums, and an alternative route for challenge in vaccine trials.

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SECTION D

Epidemiology And Vaccination Studies In Wild Possums

Introduction

The ultimate test of vaccination as a tool for the control of tuberculosis in possums was to measure its performance in a fully-wild, naturally-infected population. The site chosen for this study was at Castlepoint, on the north eastern Wairarapa coast in the North Island, NZ. The site was covered in a mixture of native bush, gorse and flax and the possum population there had been under continuous observation since 1989. Commencing in 1995, two studies were conducted on the site. The first observed the population as it re-established following localised eradication, and the second examined the efficacy of BCG in the possum population.

In 1994 the study site and the area around it was depopulated of possums. A study was made (Chapter 8) of the rate of population regeneration and the re-emergence of tuberculosis in the population. This study cover the period of 40 months immediately before the field vaccination study commenced. The temporal and spatial pattern of disease on the site was followed using restriction endonuclease analysis (REA) of the different strains. Although studies have been made of areas subjected to depopulation, none have involved a study of localised eradication.

After establishing that BCG could induce a protective response in possums held in large enclosures and that protection was enduring, the field study was undertaken (Chapter 9). The objective of the field study was to determine the efficacy of the vaccine and practicability of vaccinating a wild population with BCG vaccine delivered by intranasal aerosol and conjunctival instillation. Vaccine efficacy was evaluated using measures of incidence, determined by surveying the population every 2 months for 24 month, and prevalence, by killing and examining the population of the area at the end of the two year study.

Intranasal aerosol plus conjunctival vaccination were chosen for the field vaccination study. This enabled us to maintain a high level of control of which possum were vaccinated and when and where they were vaccinated. Intranasal aerosol and conjunctival vaccination was chosen for the field study as it had been shown as effective in the pen studies. It was chosen as an initial strategy for this exploratory level study of vaccination of wildlife under field conditions because it was the only means available that reflected possible options for

delivery of vaccine to wild possums. It was not chosen to preempt the decision on which method for delivering vaccine to wild possums, aerosol or oral, should be favoured.

There was no absolutely reliable experimental design to test the efficacy of BCG vaccination in the field on uncontrolled animals. Designs, which compare separate populations of vaccinated and unvaccinated animals, are confounded by differences in degree of exposure. We chose to stratify the site into blocks based on the historical prevalence of tuberculosis in the areas and then within each block to randomly allocated possums to vaccinated or unvaccinated groups, such that 50% of the trapped population were vaccinated at least once during the study.

Chapter 8

The re-emergence of bovine tuberculosis in brushtail possums (*Trichosurus vulpecula*) after localised possum eradication *

* Submitted as: L.A.L. Corner, M. Stevenson, D.M. Collins and R.S. Morris, to New Zealand Veterinary Journal.

ABSTRACT:

AIMS. To examine the spatial and temporal pattern of bovine tuberculosis in a re-emerging population of brushtail possums (*Trichosurus vulpecula*) after localized possum eradication.

METHODS. The possums on a 36-hectare site were eradicated and the re-emerging population was surveyed approximately every 2 months for 40 months. A capture-release regime was used and at each trapping session all possums were examined for clinical signs of tuberculosis. The diagnosis of tuberculosis was confirmed by the isolation of *Mycobacterium bovis*, and restriction endonuclease analysis (REA) was used to type the isolates. Possums which became infected were categorized as residents (present on the site for at least six months before diagnosis), range expanders (adult possums which had extended their nearby home ranges to become trappable within the site) or juvenile immigrants (sub-adult possums which had dispersed into the site from an unknown distance away).

RESULTS. Thirty cases of tuberculosis were found among the 370 possums identified on the site. Four different REA types (2, 3, 8 and 10) were identified. The first 2 cases were in range expander possums, mature males diagnosed at 4 months into the study. Case 3 was detected at 6 months. It was a female that became mature shortly before being diagnosed, and may have been a true immigrant. Case 4 was detected at 9 months and was a range-expanding mature female. Each of the first 4 cases was infected with a different REA type. The temporal pattern of infection was consistent with transmission from range-expander cases and dispersing juvenile immigrants to resident possums. Clinical incidence remained low but persistent until the third year, when types 2, 8 and 10 showed escalating clinical incidence. Type 3 showed an earlier incidence peak, but then died out. Of dispersing juvenile possums entering the site, four became clinically tuberculous and represented a source of re-infection – a high number considering the size of the site.

CONCLUSIONS. Re-emergence of tuberculosis after depopulation was due to the continuing reintroduction of infection in mature and immature diseased possums, and not the survival of *M. bovis* in the environment.

INTRODUCTION

In New Zealand bovine tuberculosis has a complex epidemiology with 14 different species of domestic and wild animals having been found infected with *Mycobacterium bovis* (Lugton, 1997). The principal wildlife reservoir is the brushtail possum (*Trichosurus vulpecula*) (Morris and Pfeiffer, 1995) which acts as a source of infection for domestic cattle and deer (O'Neil and Pharo, 1995). Tuberculosis in possums is predominantly a respiratory disease with the majority of infected possums developing extensive pulmonary lesions (Cooke et al, 1995). Paterson and Morris (1995) and Sauter and Morris (1995) described a mechanism for transmission of infection from terminally ill possums to cattle and deer. They proposed direct aerosol transmission between possums and livestock, when the livestock investigate terminally ill possums, after being attracted by the abnormal behaviour of the possums (Julian, 1981).

Culling of infected possum populations is the current strategy for controlling tuberculosis in wild possums. It is routinely undertaken where possums are considered to be the source of infection for domestic livestock. Large scale culling of infected populations results in significant reductions in the incidence of tuberculosis in cattle (Caley et al, 1999). However, where possum control is not continued the level of disease in cattle recovers to earlier levels after 8 - 10 years, even where there is annual testing of cattle herds (Barlow, 1991). Left unchecked, culled populations recover through breeding and immigration, and the number of tuberculous possums may also increase. Regular culling is therefore undertaken to maintain a low risk of infection for livestock, and possibly to eradicate tuberculosis from possum populations (Caley et al, 1999). However, simple broad scale culling of infected populations does not lead to disease eradication, because tuberculosis in possum populations is spatially clustered (Morris and Pfeiffer, 1995).

Tuberculosis in possum populations is spatially and temporally aggregated, with the spatial clusters being termed "hotspots". The size of the hotspots, based on den locations, are generally small, with a cross sectional width of 20 - 40 m (Pfeiffer et al, 1995). Some hotspots have been found to persist for 16 - 20 years (Coleman and Caley, 2000) and frequently persist in spite of population control programs (Caley et al, 1999). Pfeiffer (1994) hypothesised that the spatial component of the persistence of hotspots was due to a combination of pseudo-vertical transmission, daughters establishing a home range that overlapped with that of their mother, aggregated mating patterns and environmental

stresses. Temporal clustering, it was postulated, resulted from environmental stress, low temperatures, rainfall and poor nutrition (Pfeiffer, 1994). Hotspots may provide an environment that favours social interactions which are likely to result in tuberculosis transmission (McKenzie et al,1999)

Knowledge of the source of *M. bovis* infecting emergent populations after control should help determine the extent and frequency of subsequent control activity. Where *M. bovis* infection reappears in possum populations after culling, there are three potential sources of infection: resident infected possums surviving the cull, infected possums moving into the area, and environmental survival of *M. bovis*. The relative importance of each source has not been determined, nor has the spatial and temporal pattern of the re-establishment of infection been studied. Studies of mature possums (Efford et al.2000) have shown that once possums have established a home range they do not move far outside this range, but will expand the size of the home range if population density in adjoining areas is reduced.

In order to address these issues, possums in an area with endemic tuberculosis were eradicated, and a study was undertaken of the repopulation and the temporal and spatial patterns of tuberculosis as the disease re-emerged.

MATERIALS AND METHODS

The study was conducted on a 36 hectare site on the north-eastern Wairarapa coast at Castlepoint, as described previously (Pfeiffer 1994). The tuberculosis-infected possum population on the site had been studied intensively since 1989. During September and October 1994, all possums on the site were killed. The regeneration of the population was monitored for 40 months using a capture-release regime. Monitoring of the population commenced 1 month after the end of the eradication program, was repeated a month later and then bimonthly (Figure 8.1). Monitoring sessions consisted of trapping over three consecutive nights.

All possums in the study were individually identified with numbered metal ear tags and ear tattoos. At each trapping session, all captured possums were sedated with ketamine hydrochloride (100 mg - 150 mg; Parnell Laboratories, East Tamaki, New Zealand),

weighed and examined. The body condition (scored on a scale of 1 = emaciation, to 5 = fat) of each possum was assessed, as was its maturity and the reproductive status of mature females. A male possum was defined as mature if the testicles were >13 mm diameter and a female as mature if it had an invaginated pouch. When a pouch young was present, its sex, head length and body weight (if not attached to a teat) were recorded.

Each possum was examined for clinical evidence of tuberculosis, defined as swelling of one or more superficial lymph nodes and / or the presence of a draining sinus. The lymph nodes palpated were the parotid, mandibular, superficial axillary, deep axillary and inguinal. Material aspirated from swollen lymph nodes or collected from draining sinuses was examined bacteriologically. A definitive diagnosis was made only after the isolation of *M. bovis* from lesion material. Isolates of *M. bovis* were typed using restriction endonuclease analysis (REA; Collins, 1999). Possums found with clinical tuberculosis during the first year of the study were fitted with radio collars to determine the location of their den sites and to recover their bodies after death.

Opportunistic sampling of the population was achieved by *post mortem* examination of possums that died during trapping, were found dead, or were killed by trappers. Post mortem diagnosis of tuberculosis depended on the isolation of *M. bovis* from animals with macroscopic lesions or from a pool of tissues collected from animals with no macroscopic lesions. The pool consisted of the superficial axillary, deep axillary, and inguinal lymph nodes, liver, spleen and lung.

Possum abundance (population size) was estimated for each session using the Jolly-Seber algorithm (Seber, 1982). For the purposes of analysis, mature possums which extended their nearby home ranges to become trappable within the site were categorized as "range expanders", and juveniles independent of their mother were classified as "juvenile immigrants" if they were recorded as being on the study site for less than six months before lesions of tuberculosis were detected. Possums that had been trapped on the site for at least six months before being detected as tuberculous were classified as "resident".

Details of the geographic location of each possum captured was determined using a global positioning device and recorded with the capture details described above. Spatial distribution of traps where tuberculous possums were captured and the dens used by tuberculous possums were plotted using ArcView for Windows version 3.1

(Environmental Systems Research Institute, 1998). The hotspot for each REA type was constructed using the Gaussian kernel density estimator in the Spatial Analyst Extension version 1.1. in ArcView. A hotspot was defined for each REA type as the area within a contour line that enclosed 75% of all captures for animals known to be infected with that REA type. A fixed bandwidth of 0.0004 degrees was used and was calculated by the procedure of normal optimal smoothing (Bowman and Azzalini, 1997).

We sought to determine if there were areas within the study region where there was an excess of tuberculous possum density when compared with the spatial density of all possums that were captured. Two density surfaces were constructed: the first for tuberculous possums (cases) and the second for all possums that were caught throughout the study period (population). The ratio of the kernel density surface for the cases to the kernel density surface for the population provided a relative risk surface that identified, at each point in the study region, the proportion of tuberculous possums per unit of captured possum density (Bithell, 1990; Lawson and Williams 1994, 2001). To more formally identify areas of tuberculosis excess, while correcting for the spatial distribution of all possums that were caught, we determined the difference between the square root of the density estimate computed for the cases and for the population. After Bowman and Azzalini (1997), a standardised density difference greater than two in absolute value was arbitrarily chosen to delineate areas of excess or decreased tuberculosis density.

RESULTS

Initially the population on the site built up rapidly and at the end of 6 months was estimated to be 104, reaching a maximum of 167 at 30 months (Figure 8.1). During the study, 370 individual adult possums were captured. Over the 40 months, 22 of the 370 possums were found dead on the study site of which 18 were examined post mortem. Trappers killed 49 possum of which 26 were examined post mortem.

The temporal pattern of incident cases and the number of known prevalent cases are shown in Figure 8.1. Prevalent cases were those tuberculous possums known to be alive at a particular trapping session, either having been trapped at that or later session(s). The time of death of tuberculous possums was determined by radio tracking (which was done at weekly intervals), and other possums not subsequently caught were deemed to have died soon after the last time they were captured. Cases of tuberculosis were first recorded at 4

months, then intermittently until 30 months, after which there was a dramatic rise in the number of new cases, a level that was sustained until the end of the study (Figures 8.1 and 8.2).

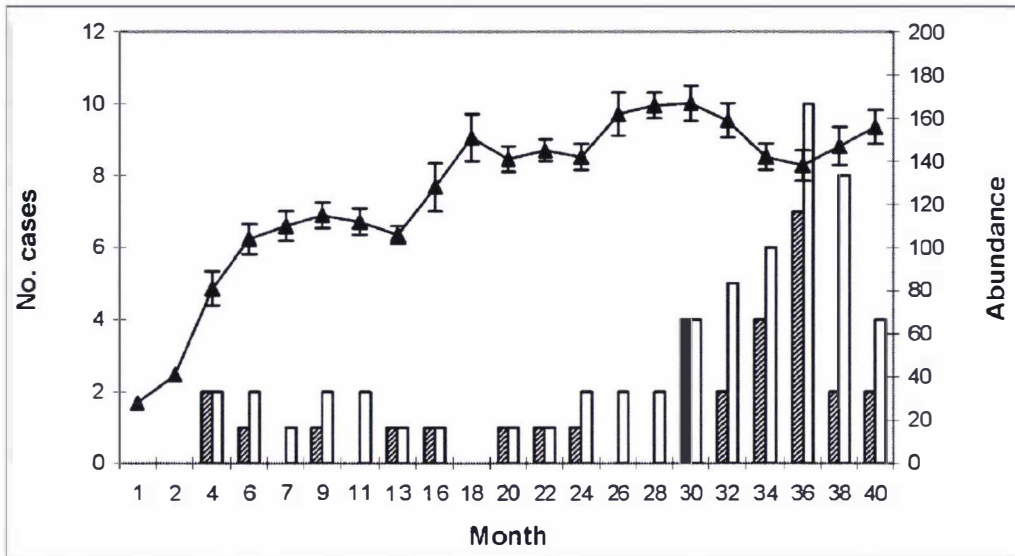


Figure 8.1

The temporal pattern of tuberculosis in possums during the repopulation. Incident cases (cross hatched), prevalent cases (white) of tuberculosis and population abundance estimates (line) with \pm 95% confidence intervals (bars) at each trapping session.

Thirty cases of tuberculosis were identified during the study, of which 28 were first diagnosed by clinical examination and two, that did not have clinically detectable lesions, were diagnosed by post mortem examination. Of the two tuberculous possums that did not show ante mortem clinical signs, one was found dead on pasture and the other was killed by a trapper. Four REA types were identified in the 30 isolates of *M. bovis*, of which 13 were type 2, four were type 3, six were type 8, six were type 10, and one isolate was not available for typing (Figure 8.2).

Over the course of the study 10 possums with tuberculosis were classed as non-residents when tuberculosis was first diagnosed. Six were range expanders (4 mature males, and 2 mature females), and four were juvenile immigrants (2 immature males, and 2

immature female). Of the 20 resident possums diagnosed with tuberculosis, 13 were mature males, 5 were mature females, and there was one immature male and one immature female. In the first year 3 of the tuberculous possums were range expanders and one was a juvenile immigrant. In the second year there were 5 cases, 4 in residents and one in a juvenile immigrant, an immature male. In the final 16 months, there were 21 cases, 16 were in residents and of the remaining 5, 3 were range-expander adults and 2 were immigrant juveniles that included one immature male and one immature female.

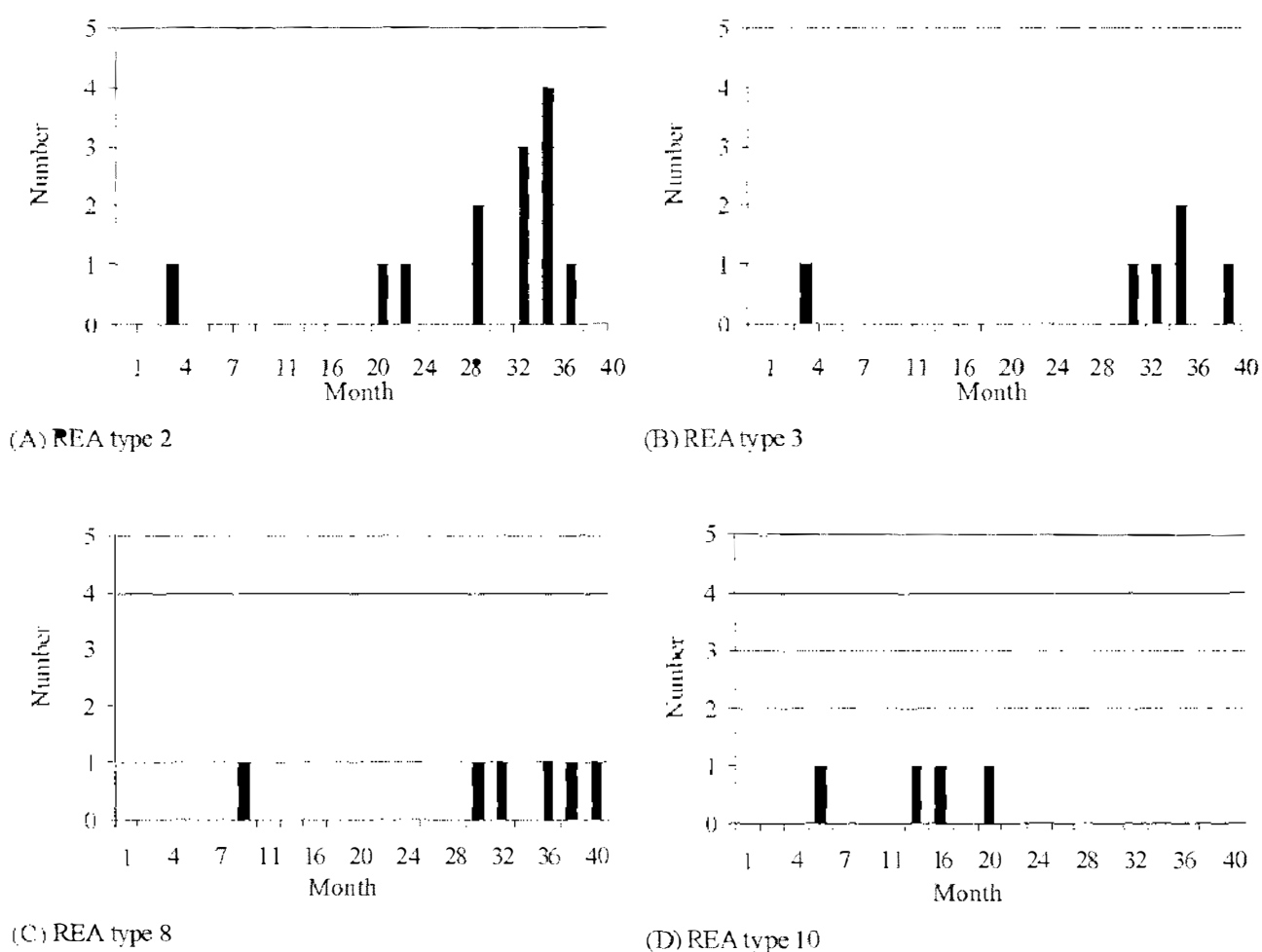


Figure 8.2

The temporal pattern of tuberculosis cases during a study of the efficacy of BCG vaccination in wild brushtail possums. Shown are the number of incident cases for each restriction endonuclease analysis type at each trapping session: A) REA type 2, B) REA type 3, C) REA type 8 and D) REA type 10.

Capture locations and frequency of capture were used to describe the spatial distribution of tuberculous possums for each REA type and to identify the hotspots of infection for each type (Figure 8.3). The hotspots for REA types 8 and 10 were similarly located and partially overlapped with the larger REA type 2 hotspot. The REA type 3 hotspot was adjacent to the other 3 hotspots. The vegetation in the hotspots was diverse (flax, gorse, manuka and pasture), and the topography ranged from ridges and steep slopes to valley floor.

There were different temporal patterns for each hotspot. The hotspot associated with REA type 3 was present only between 6 and 20 months, when the 4 cases infected with this type were alive. The western hotspot associated with REA type 2 became apparent at 22 months and the central area at 30 months. The hotspot associated with REA type 8 became apparent at 32 months and that due to REA type 10 at 34 months.

For each REA type hotspot(s) were defined as the area where the top 75th percentile of captures occurred (Figure 8.3A to 8.3D). In Figures 8.3A to 8.3D the inner contour represents the top 25th percentile, the middle contour the top 50th percentile and the outer contour the top 75th percentile.

The histories of the first six cases

Case 1 – A mature male (H5724) infected with REA type 2. It was first diagnosed with tuberculosis at 4 months when trapped on the western boundary (Figure 8.3A). It was trapped in an area that became one of 2 hotspots for REA type 2 and was found denning nearby. It was clinically normal when first trapped 2 months earlier and was last seen at 10 months.

Case 2 – A mature male (H5780) infected with REA type 10. This possum was trapped only once, at 4 months (Figure 8.3B), on the northern boundary and 350 m from the centre of where the REA type 10 hotspot developed.

Case 3 - A mature female (H5718) infected with REA type 3. She was immature and clinically normal when trapped at 2 and 4 months. When trapped at 6 months she had reached maturity and was diagnosed with tuberculosis. On that occasion she was trapped on the northern boundary, 100 m from the centre of where the REA type 3 hotspot developed (Figure 8.3C). Over all she was trapped four times about 100 m from where the

REA type 3 hotspot formed but was trapped once within the area of the hotspot, and also found to den there. She died 2 weeks after diagnosis.

Case 4 - A mature female (H5620) infected with REA type 8. Tuberculosis was first diagnosed at 9 months when she was trapped deep within the study site (Figure 8.3D). She had been clinically normal when first seen at 4 months and died 2 months after diagnosis. She was trapped and denned 250 m from the centre of where the REA type 8 hotspot formed.

The fifth and sixth cases were in resident mature males diagnosed at 13 and 16 months respectively, and both were infected with REA type 3.

The spatial distribution and density of sites where tuberculous possums were trapped is shown in Figure 8.4B and for all possums in Figure 8.4A. In Figure 8.4A, the “25” refers to the top 25th percentile and the contour encloses an area where the estimated trapping density was greater than 375 catches per ha over 3 years (54 trapping occasions). The top 50th percentile (“50” contour) encloses the area where it was greater than 138 catches per ha over 3 years and the top 75th percentile (“75” contour) an area with greater than 3 catches per ha over 3 years. For Figure 8.4B for the top 25th percentile the density of captures was greater than 300 catches, for the top 50th percentile greater than 45 catches and for the top 75th percentile greater than 0.06 catches.

From the spatial density estimates for the entire population (Figure 8.4A) and that for the tuberculous possums (Figure 8.4B), the estimated relative risk surface was calculated (Figure 8.4C). The standardised density areas (2 standard differences above or below that estimated for the population density; Figure 8.4D) showed where the density of diseased possums exceeded that expected (+2, tuberculosis hotspot) and areas where it was below the expected (-2).

DISCUSSION

Tuberculosis reappeared on the study site soon after the repopulation commenced. There were 2 cases at 4 months and additional cases at 6 months and 9 months. Three of the possums were trapped on the periphery of the site and the fourth in the centre of the site. The mature possums appeared to have extended their ranges into the vacated area and the immature possum probably entered during juvenile dispersal (Efford et al,

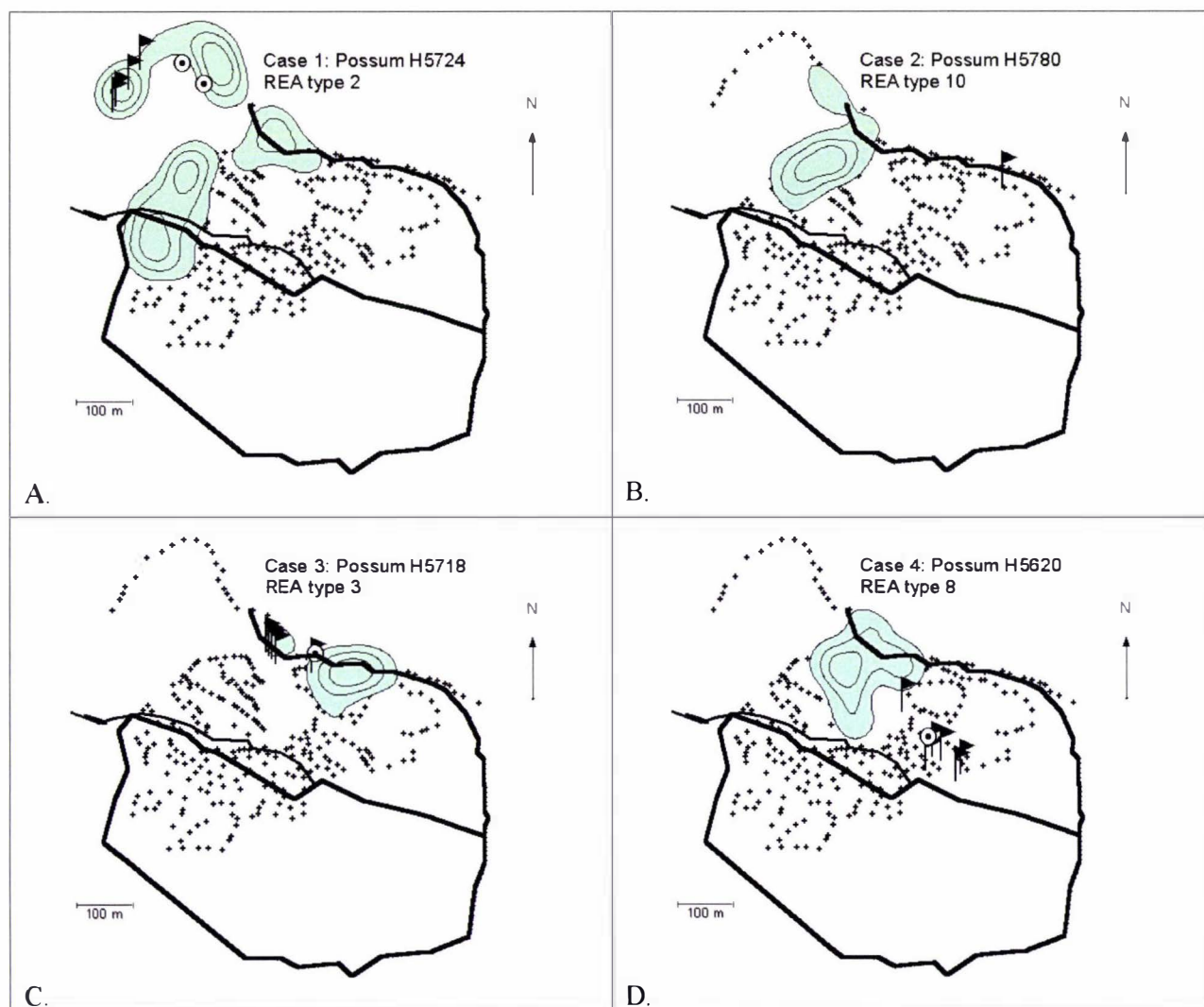


Figure 8.3

The capture locations and den sites for the first four tuberculosis cases and the spatial distribution of tuberculosis cases infected with each REA type (hotspots). Trap (triangular flags) and den sites (annuli) are shown for the first 4 tuberculous possums. The inner contour encloses the area of highest density of captures of tuberculous possums (top 25th percentile), the middle contour the top 50th percentile and the outer contour top 75th percentile. Small crosses mark the location of traps site and lines the location of fences, tracks and creeks.

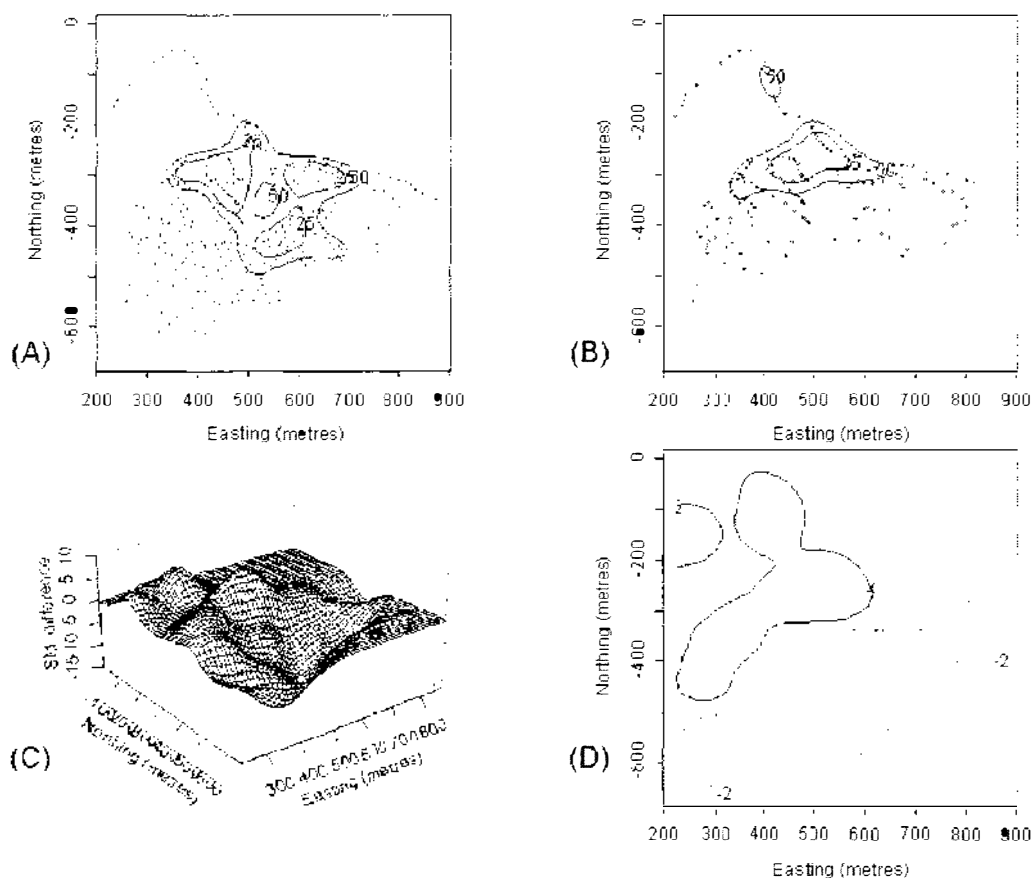


Figure 8.4

Tuberculosis hotspots on the study site calculated using relative risk in two dimensions. The spatial density estimates for the frequency of trapping tuberculosis possums (Figure 8.4A) and trapping of all possums (Figure 8.4B); the estimated relative risk surface (Figure 8.4C), and the standardised density differences (Figure 8.4D). In Figures 8.4A and 8.4B, the inner contour encloses the area of highest density of captures, top 25th percentile, the middle contour the top 50th percentile and the outer contour top 75th percentile.

2000). It may well have been a pseudo-vertically infected animal. Two of the 4 were trapped or denned in areas that subsequently became hotspots for the same REA type.

The clinical incidence of tuberculosis remained low during the first 2 years, but diseased animals of at least one REA type were present almost continuously throughout this period. There was an upsurge in the incidence from 30 months onwards and this followed the peak in the population size at 28 - 30 months. The disease prevalence peaked

at 36 months. For 3 of the 4 REA types there was a long interval, 18 to 25 months, between the first and subsequent cases, whereas for type 3 the second case occurred six months after the first, and was followed by two more cases in quick succession. This type then disappeared entirely for the remaining 18 months of the study. For all REA types, one or more animals affected in the second wave of cases were present on the site when the index case for that type was diagnosed, so transmission could have occurred at that time. Infected juvenile immigrants probably strongly influenced the epidemiology of types 2 and 3, whereas infection with types 8 and 10 appeared to result entirely from range expanders.

Lugton (1997) has described REA findings from the study site and the surrounding district over the period prior to the depopulation, which can be compared with events during the repopulation. The four REA types found after recolonisation were four of the five types most commonly found in the district (82% of 130 possum isolates tested) and on the study site (89% of 113 possum isolates tested). Types 2 (38% of possum isolates from the site) and 3 (35%) were the dominant REA types on the study site through most of the 66 months prior to depopulation. Type 2 had initially been common but dwindled to a low level and then underwent a major resurgence just prior to the depopulation, whereas type 3 was common for four years, then disappeared entirely for 21 months prior to the depopulation, and was rediscovered at that time in untagged animals living just to the north of the study site area. Type 8 was an uncommon type on the study site, but was common 1 to 3 kilometres to the south of the study site, and had only been found in two juveniles on the study site five years before the depopulation. It appears to have re-established on the site from its primary nidus during the repopulation of the site. Type 10 appeared to have a nidus just to the west of the study site, and had caused intermittent cases on the study site for five years prior to the depopulation. It has never been found further afield from the study site.

Infection became highly prevalent in the population after 30 months, following the stabilisation of the population, and the high number of cases then continued for 6 months. Three of the 4 REA types, plus an untyped strain, were involved in the upsurge in cases at that time. In the final 2 years, the proportion of non-resident possums among the cases remained around 25%. As that proportion remained reasonably constant in the last 12 months of the study, it suggests that whatever caused the upsurge in tuberculosis on the

study site may have caused a similar upsurge in the surrounding area where there had been no control of the possum population.

Each of the REA types found on the site had its own epidemiological pattern and each was associated with its own hotspot(s) of infection. Through the use of REA it was possible to divide the general re-emergence of infection into separate epidemiological patterns due to each type. While there was almost continuous presence of infection on the site for the duration of the study, there were in fact four separate patterns overlapping in time and space. For each REA type, animals which later became tuberculous with that type were present on the site when the index case was found. It would appear that infection may have been transmitted from the index case, and became evident in the resident population from months to well over a year later.

We observed a high degree of spatial and temporal clustering of tuberculous possums in the infected population. Clustering has been reported previously both at Castlepoint (Pfeiffer 1994) and elsewhere (Coleman and Caley, 2000). The hotspots due to each REA type overlapped to a large degree, especially those due to REA types 2, 8 and 10. The hotspots for the different REA types developed at different times and that due to REA type 3 disappeared when the last case infected with this REA type died at 20 months. The central common area was consistent with a hotspot described by Jackson (1995). Although the hotspots were near where the possum population was concentrated, they were not coincident with the population concentrations.

It has been postulated (Morris and Pfeiffer, 1995) that the spatial clustering is due to physical features of the environment that indirectly affect the frequency of infectious interactions between possums, and is not directly due to environmental survival of *M. bovis*.

The pattern of infection on the site demonstrated that environmental contamination was a minor, and probably insignificant, source of infection for possums. Experimental studies have demonstrated that *M. bovis* survives in the environment for only a few days to a few weeks (Jackson et al. 1995). The pattern of infection on the site after recolonisation was consistent with infection being maintained in animals, not in the environment. The one common REA type (Lugton type C) which did not re-establish after depopulation had been the second most common immediately prior to depopulation, yet was completely

extinguished by the depopulation process, whereas types 3 and 8, known to be in the vicinity but absent from the study site for one to five years before depopulation, successfully re-established during recolonisation of the site.

After control operations, possum populations recover through a mixture of breeding of survivors, recruitment of their offspring, and recruitment of both juvenile and mature possums from the surrounding area. In the early post-control period inward migration will be more important than breeding. Recruitment from the local surrounding area is of two types. Short distance movements, of up to 200 - 300 m, are made by mature possums whose home ranges border the controlled area (Efford et al, 2000). Recruitment of immature possums includes some from further afield as a result of the long distance movements undertaken during juvenile dispersal. These long distance movements are undertaken by about 20% of juveniles who move on average 2 to 10 km but may exceed 20 km (Cowan and Clout, 2000). Although juvenile males show a greater propensity to disperse over long distances, juvenile females tend to move further (Cowan et al, 1996). The inference from detecting tuberculosis in a mature range-expander possum was that a hotspot existed close to the boundary of the study site. However, no such inference regarding the location of a hotspot could be made from the detection of disease in juvenile possums. Recolonisation of this site with tuberculosis appears to have been due initially to both range-expander adults and juvenile immigrants, with infected juveniles continuing to arrive throughout the study.

It is apparent that not all tuberculous possums readily transmitted infection to other possums. Cases infected with REA type 3 were seen in a 14 month period and not subsequently. Transmission will be influenced by contact rates, which in turn are influenced by the population density, duration of survival of the infected possums and the social position of the affected possums.

The results strongly support the view that infection of the emerging population on the site was due initially to inward migration of infected possums. Apart from environmental survival of *M. bovis*, the other possible source of infection was that one or more tuberculous possums survived the depopulation. This is an unlikely explanation as only 3 possums from the previous population were found during the current study and all were free of tuberculosis. The probability of other possums surviving is remote, let alone 1 or more infected animals. However, following routine culling programs, where many more

possums survive, infected survivors may be a significant source of reinfection, particularly in long-term hot spots.

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Chapter 9

The efficacy of bacille Calmette-Guérin vaccine in wild brush-tail possums (*Trichosurus vulpecula*)*

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ABSTRACT:

A population of wild brushtail possums (*Trichosurus vulpecula*) in which bovine tuberculosis was endemic was vaccinated with live bacille Calmette-Guérin (BCG) to determine the efficacy of the vaccine. The population on a 56 hectare site was monitored bimonthly over 2 years using a capture-release regime. During the study tuberculosis was diagnosed by clinical and post mortem examination. Possums were vaccinated with bacille Calmette-Guérin by both intranasal aerosol and conjunctival instillation. Possums were revaccinated on average every 5 months. Over the 2 years 300 possums were recruited to the study, with 149 being allocated to the vaccination group. There were significantly fewer cases of tuberculosis in the vaccinated (4 cases) than in the unvaccinated group (13 cases; $p = 0.023$). The vaccine efficacy was 69%. An attempt was made to increase the incidence of disease by releasing experimentally infected possums onto the site. However, this did not result in any additional cases. BCG vaccine was shown to have a level of efficacy which could be of assistance in controlling tuberculosis in wild possum populations. The future use of vaccination for the control of tuberculosis in wild possum populations is discussed.

INTRODUCTION

Bovine tuberculosis in wild animals is becoming a world wide problem, particularly where they act as reservoirs of infection for domestic animals, other wild animal species and humans. Wild animal reservoirs have been identified in cervids in North America (Schmitt et al 1997), badgers in the United Kingdom and Ireland (O'Reilly and Daborn 1995, Hughes et al 1996), African buffalo in South Africa (O'Reilly and Daborn 1995), and brushtail possums (*Trichosurus vulpecula*) in New Zealand (Morris et al 1994). Vaccination of wild animals may be the only alternative to culling as a means of controlling infection in wild populations. Vaccination would be preferable to culling for wild animal species of high economic or high conservation value (Hughes et al 1996) and may be a more affordable means of eradicating tuberculosis in wildlife than continued culling.

The brushtail possum is the major wildlife reservoir of *Mycobacterium bovis* in New Zealand (Morris and Pfeiffer 1995). Infection is endemic in possum populations across 33% of the country (Animal Health Board 2001). Tuberculosis in possums is controlled by

culling infected populations to reduce the risk of transmission of infection to domestic stock, but culling alone does not eradicate the disease (Caley et al 1999, Animal Health Board 2001). Culling removes the majority of possums in the target area, but some tuberculous possums survive to maintain the disease. The disease in possum populations is clustered spatially and temporally (Morris and Pfeiffer 1995), which partly explains the maintenance of disease despite extensive culling. Culling is costly and an unending process, for without continued culling populations rapidly recover and prevalence of tuberculosis with it. For long term control, new strategies are required. Vaccination of infected possum populations is a promising option (Buddle et al 2000)

Vaccination of captive possums with bacille Calmette-Guérin (BCG) has been shown to provide protection against intratracheal challenge with *M bovis* (Aldwell et al 1995a, Buddle et al 1997, Corner et al 2001a). Vaccinated possums had less severe disease and less dissemination of infection from the lungs to the liver and spleen. Possums have been successfully vaccinated by intranasal aerosol (Aldwell et al 1995a), subcutaneous inoculation (Aldwell et al 1995a), intratracheal instillation (Aldwell et al 1995b), intraduodenal instillation (Buddle et al 1997) and conjunctival instillation (Corner unpublished observations), but oral administration was ineffective (Aldwell et al 1995a).

BCG vaccination has attributes that make it an attractive disease control strategy for infected possum populations. A single dose of vaccine produced a protective effect that was still demonstrable after 12 months (Corner et al 2001a) and multiple doses over a short time period increased the level of protection (Corner unpublished observations). Vaccination could be used alone or in conjunction with other control measures. Modelling of tuberculosis in possum populations predicts that the prevalence of disease could be reduced, and possibly eradicated, using a vaccine (Roberts 1996). Given that an effective delivery device can be developed, aerosol vaccination of wild possum populations could be a suitable method for controlling tuberculosis.

The aim of this study was to evaluate the efficacy of BCG vaccination in a wild possum population in which tuberculosis was endemic. Vaccine efficacy was assessed by comparing the incidence of disease in vaccinated and unvaccinated groups. The study was conducted over 2 years at a site where epidemiological research into bovine tuberculosis in wild possums had been maintained continuously for 10 years.

MATERIALS AND METHODS

Experimental design

A population of wild brushtail possums in which tuberculosis was endemic was selected for the study. The population was examined bimonthly using a capture-release regime. Tuberculosis was diagnosed by clinical examination or post mortem examination with the diagnosis confirmed by culture of *M bovis*. Approximately 50% of the possum population was vaccinated. At the end of the study all possums on the site and within a 300m zone surrounding the site were killed and examined.

In an attempt to increase the incidence of tuberculosis on the study site, eight resident possums were artificially infected and released back onto the site. Mature males were used as they range more widely and are believed to engage in more frequent social interactions than females, especially during the breeding season (Day *et al* 2000).

Study site

The 56 hectare study site was on the north eastern Wairarapa coast of the North Island of New Zealand and had on it 450 cage traps in fixed locations. Vegetation consisted of native scrub, remnant forest and pasture. The possum population in the area surrounding the site was subject to a culling operation shortly before the study commenced (February 1998) as part of a regional possum control program. To minimise the effects on the study population, the culling operation did not come closer than 300m to 400m from the site boundary.

Trapping sessions to monitor the possum population were conducted bimonthly from February 1998 to December 1999. At each session the traps were set on 3 consecutive nights. Possums were individually identified with numbered metal eartags and ear tattoos.

Possum examination

The first time a possum was trapped during a session it was sedated and examined, and if allocated to the vaccinated group, was vaccinated. Possums were sedated with 100 to 150 mg ketamine hydrochloride given by intramuscular injection. The data collected included weight, assessment of body condition (scale 1 = emaciated, to 5 = very fat), and estimation of age based on surface wear of the first dorsal molar (scale 1 = pouch young,

no apparent wear, to 7 = crown dished, Winter 1980). Males were classed as mature if the testicles were >13 mm diameter, and females as mature if their pouch was invaginated. The reproductive status of females was determined by evidence of lactation, presence of pouch young or of a juvenile backrider.

All possums were examined for clinical signs of tuberculosis by palpation for enlargement of the superficial axillary, deep axillary, inguinal, parotid and mandibular lymph nodes, or identification of draining sinuses. When a lesion suggestive of tuberculosis was detected a tissue specimen, lesion aspirate or swab was collected for bacteriological confirmation. Each possum found with clinical signs of infection was fitted with a radio collar. These possums were monitored weekly by radiotelemetry to determine survival duration following diagnosis of tuberculosis and to enable their bodies to be recovered for detailed necropsy.

Allocation to treatment groups

To ensure that no bias was introduced into the allocation of possums to treatment groups, a selection process was used that accounted for the spatial heterogeneity of diseased possums on the site (Jackson 1995). Possums were assigned to a treatment group based on the trap where they were first captured during the study. The area covered by traps on the site was divided into 11 sub-areas using historical records of the location and frequency of trapping of tuberculous possums. Within each area when possums were caught for the first time during the study they were allocated alternately to the vaccinated group or unvaccinated group. Pouch young were allocated to the same treatment group as their mothers since we were unable to ascertain if BCG infection was naturally transmitted to pouch young from vaccinated mothers.

Vaccination

Two routes of administration, intranasal aerosol and conjunctival instillation, that exposed both the nasal and conjunctival mucous membranes were used jointly to ensure effective vaccination (Buddle et al 1997, Corner unpublished observations). Each possum was vaccinated by both routes. Intranasal aerosol vaccination was administered as a metered aerosol spray (Valois Spray atomisers, Douglas Pharmaceuticals Limited, Auckland) of 100 µl directed at each nostril from a distance of 1 to 2 cm. Conjunctival vaccination was performed using an eye dropper that delivered approximately 50 µl of

bacterial suspension per drop. One drop of vaccine suspension was instilled into the conjunctival sac of each eye. The BCG vaccine suspension contained approximately 5×10^6 colony forming units (cfu)/ml (that is, the dose per animal was 1.5×10^6 cfu). The vaccine was prepared as described by Aldwell et al (1995a). Vaccinated possums were re-vaccinated every 4 months, or as close to that time interval as possible, depending on the frequency with which possums were trapped.

Lymphocyte proliferation assay (LPA)

The immunological response to vaccination was monitored with the lymphocyte proliferation assay, performed as previously described (Cooke et al 1999). Surveys of the population were conducted in July and November of the second year. Blood samples were collected by cardiac puncture from both vaccinated and control possums. The stimulation index to bovine purified protein derivative (PPD: bovine SI) was calculated as the mean response to bovine PPD divided by the mean of the unstimulated control wells.

Artificial infection

Eight mature male possums were trapped on the site and experimentally infected with a strain of *M bovis* that had a rare restriction endonuclease type (REA type LF). Three possums were infected in August 1998, 3 in February 1999 and 2 in August 1999. Following sedation the possums were anaesthetised with 12 mg of Saffan (Pet Elite Ltd, Lower Hutt, NZ) administered by intramuscular injection. An endotracheal cannula was introduced *per os* and passed down the trachea to the level of the bifurcation. When in place 200 μ l of *M bovis* suspension was instilled into the lungs (Pfeffer et al 1994). The inoculum contained approximately 100 cfu. Before being released each infected possum was fitted with a radio-collar so that their survival time could be determined and the cadaver recovered for post mortem examination.

Depopulation of the site at the end of the study

Over a 6 week period in February and March 2000, all possums on the site and within 300m of the site boundary, were humanely killed and subjected to a detailed post mortem examination.

Post mortem examination

During the study there was opportunistic sampling of the population by the *post mortem* examination of possums that died during trapping, were euthanased, or were killed off-site by trappers. Tuberculosis was diagnosed at necropsy by detection of characteristic macroscopic lesions and lesion material was collected for bacteriological confirmation. All post mortem examinations were conducted using a standardised procedure. At the examination data was collected on the animals sex, maturity, body weight and reproductive status of mature females. The post mortem included visual examination of head, body and abdominal lymph nodes and abdominal organs. Macroscopic granulomatous lesions indicative of tuberculosis were collected for bacteriological examination and a tracheal swab was collected from possums with macroscopic lung lesions. Where no macroscopic lesions were found the following tissues were pooled for bacteriological examination: superficial axillary, deep axillary, inguinal, and mesenteric lymph nodes, and sections of lung, liver, and spleen.

The post mortem examinations at the depopulation were more detailed than those done during the study. The pool of tissues for bacteriological examination from possums with no macroscopic lesions consisted of superficial axillary, deep axillary, inguinal, hepatic and mesenteric lymph nodes, and sections of spleen, lung and liver. In addition tissues were collected from all possums for histological examination. The tissues for histopathology included the mandibular, parotid, retropharyngeal, caudal cervical, superficial axillary, deep axillary, inguinal, mesenteric, gastric, hepatic and tracheobronchial lymph nodes, and spleen, liver, kidney, adrenal glands and lungs. Pouch young were also examined for lesions and liver, lung and spleen were collected for bacteriological and histological examination.

Bacteriology

Primary isolation of *M bovis* (Corner et al 1995) and the identification of mycobacterial isolates (de Lisle and Havill, 1985) were as previously described. Isolates of *M bovis* were typed by restriction endonuclease analysis (REA; Collins 1999).

Data analysis

Vaccine efficacy was defined as the disease rate in the unvaccinated group minus the disease rate in vaccinated group divided by disease rate in the unvaccinated group (Martin et al 1988). The differences between treatment groups in the incidence of infection were compared using Fisher's exact test and differences in the LPA responses were compared using the *t* test. The size of the study populations (possum abundance) at each trapping session was estimated from trapping records, using the Jolly-Seber algorithm (Seber 1982).

The geographic location of each possum captured was determined using a global positioning device and recorded with the capture details described above. The distribution of captures of all possums and all the naturally infected tuberculous possums was plotted using a Gaussian kernel density surface in Spatial Analyst Extension Version 1.1. of ArcView for Windows version 3.1 (Environmental Systems Research Institute, 1998). The outer contour contains the traps where possums were captured 80% of the time. A fixed bandwidth of 0.0004 degrees was used, calculated using normal optimal smoothing (Lawson and Williams 1994, 2001).

RESULTS

Of the 308 individual possums recruited to the study, 149 were allocated to the vaccinated group and 151 to the unvaccinated group. Eight possums were not allocated to either group because they were found to have tuberculosis when first encountered (7 possums) and one was not allocated because no vaccine was available at the time and it was not seen again. The estimated size of the study population (possum abundance) was determined at each trapping session (Table 9.1, Figure 9.1). Also shown in Table 9.1 are the number of possums from each treatment group that were examined and the number of possums that were vaccinated or re-vaccinated. The estimated possum population at the commencement of the study was 163 and it steadily declined to 76 at the conclusion. At each session approximately equal numbers of the vaccinated and unvaccinated groups were trapped. The median number of times each possum was vaccinated was 2.3 (range 1 to 6, Table 9.2).

Tuberculosis cases

Tuberculosis was diagnosed in 24 possums from the study population. Nineteen were detected by clinical examination, 2 without clinical signs (P0041 and P0184) were found

with macroscopic lesions at post mortem and 3 without macroscopic lesions were detected at the depopulation by bacteriological examination. Except for a possum found moribund in a trap and a second that died 2 weeks after diagnosis, the possums, both vaccinates and non-vaccinates were in good physical condition (score 3 or 4) when tuberculosis was first diagnosed. The vaccinated group showed the same seasonal patterns of body weight change, level of reproductive performance and frequency of trapping as the unvaccinated possums.

TABLE 9.1

BCG vaccination of wild possums: estimated possum abundance on the study site and the number of possums examined and vaccinated at each of the bimonthly monitoring sessions.

Trapping session	Estimated Abundance*	Treatment group			No. Vaccinated or re-vaccinated
		Unvaccinated	Vaccinated	Not allocated	
Feb 98	163	66	66	4	67
Apr 98	164	71	77	5	17
Jun 98	135	71	73	2	62
Aug 98	100	69	62	1	4
Oct 98	115	59	56	1	3
Dec 98	115	51	48	0	48
Feb 99	94	55	55	0	13
Apr 99	95	43	40	1	32
Jun 99	87	43	44	0	13
Aug 99	64	47	44	0	33
Oct 99	82	34	34	0	8
Dec 99	76	32	40	1	30
Feb 00	76	39	36	1	NA**

* Possum abundance was calculated from trapping records and estimated using the Jolly - Seber algorithm (Seber)1982.

** NA - not applicable

TABLE 9.2**Frequency of vaccination of possums in the vaccinated group. (Median = 2.3)**

Frequency of vaccination	Number of possums
1	66
2	42
3	10
4	11
5	7
6	13

Seven possums that had clinical disease at the time they entered the study were excluded from the analyses. Seventeen cases were considered to be new infections arising during the course of the study. Thirteen cases were in unvaccinated possums and four were in vaccinates. The difference is statistically significant (Fishers exact test, one tailed, $p=0.023$). The relative risk of tuberculosis in unvaccinated possums compared with vaccinates was 3.21 (95% CI 1.07 to 9.61). The vaccine efficacy was 69% (95% CI 7% to 90%).

All the vaccinated possums were mature when first diagnosed with tuberculosis, as were 10/13 unvaccinated possums (Table 9.3). One possum had been vaccinated twice, two had been vaccinated three times and the fourth had been vaccinated four times.

The temporal pattern of incident and prevalent cases is shown in Figure 9.1 and the spatial distribution of tuberculous possums in Figure 9.2. Four different REA types caused tuberculosis in the possums. The temporal distribution of new cases of infection due to each REA type is shown in Figure 9.3.

During the study, but not including the depopulation, 94 possums from the study population, which had no history of clinical tuberculosis, were examined post mortem. Only two were found with tuberculosis: one was found moribund in a trap and the other was killed when found beside its dead tuberculous mother. Trappers killed 92 possums in the area surrounding the study site but none had lesions of tuberculosis. Bacteriological examination of pooled tissues from 63 of the 92 possum was negative for *M bovis*.

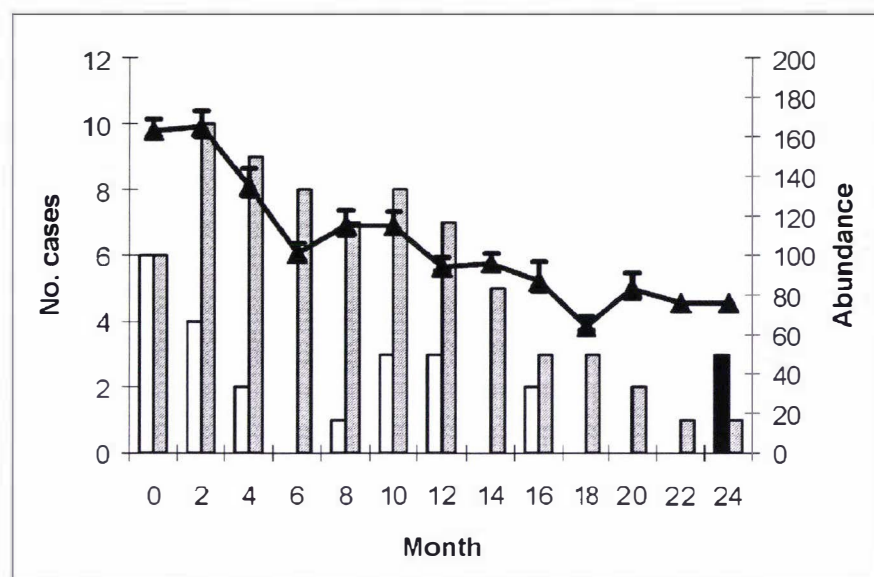


Figure 9.1

The temporal pattern of tuberculosis cases during the study of the efficacy of BCG vaccination in wild brushtail possums. Shown are the number of incident cases (white), prevalent cases (cross hatched) and the estimated population abundance (line) at each trapping session (month). The black bar at 24 months represents possums found infected when the site was depopulated.

Site depopulation

During the site depopulation, 126 possums were killed and examined post mortem. Of these 76 were part of the study population and 50 were new possums trapped on the site or in the surrounding area. Of the 76 that were from the study population, 39 were from the vaccinated group, 36 were from the unvaccinated group and one had not been allocated to a group. *M. bovis* was isolated from 3 of the study population possums, 2 from the unvaccinated group and 1 from the vaccinated group; none had macroscopic or microscopic lesions. An immature male, new to the site, had a macroscopic lesion of tuberculosis in the mesenteric lymph nodes.

Lymphocyte proliferation assay

Two surveys of the immunological responsiveness of possums to bovine PPD were conducted (Table 9.4). The mean bovine SI of the vaccinated possums was significantly greater than the unvaccinated possums at both bleeds (Table 9.4). There was no correlation between the bovine response and the number of times a possum had been vaccinated.

TABLE 9.3

The age at diagnosis and sex of possums found infected with *M bovis* by clinical or post mortem examination.

Group	Sex and Age *				Total
	FI	FM	MI	MM	
Not vaccinated	2	7	1	3	13
Vaccinated	0	2	0	2	4

FI = female immature, FM = female mature, MI = male immature, MM = male mature

Artificial infections

Eight mature male possums from the study site were artificially infected and released back onto the site. The possums survived 8 - 21 weeks. Two possums had developed palpable lesions in superficial axillary lymph nodes by 6 weeks and 13 weeks after infection and one also developed a draining sinus. Seven of the 8 possums were examined post mortem. All had extensive lung lesions and 5 had macroscopic lesions in superficial lymph nodes. One was too decomposed to ascertain lesion distribution. Tracheal swabs taken from the 7 possums were positive for *M bovis*.

DISCUSSION

Vaccination with BCG significantly reduced the risk of tuberculosis infection in the vaccinated possums. The vaccine had an efficacy of 69% and the non-vaccinated possums had a 3.23 times higher risk (relative risk) of infection when compared with vaccinated

possums. The calculation of vaccine efficacy and relative risk was based on the number of new cases in the population, where half the population had been vaccinated. New cases were detected by clinical examination for lesions of tuberculosis and post mortem examination of animals dying during the study. It would appear that vaccination prevented infection, as well as the development of disease. Griffin et al (1999) presented data supporting their claim that BCG prevented infection in experimentally infected deer. Vaccination of possums did not result in any side effects. The vaccinated group showed a similar seasonal patterns of body weight change, high level of reproductive performance and frequency of trapping as the unvaccinated possums.

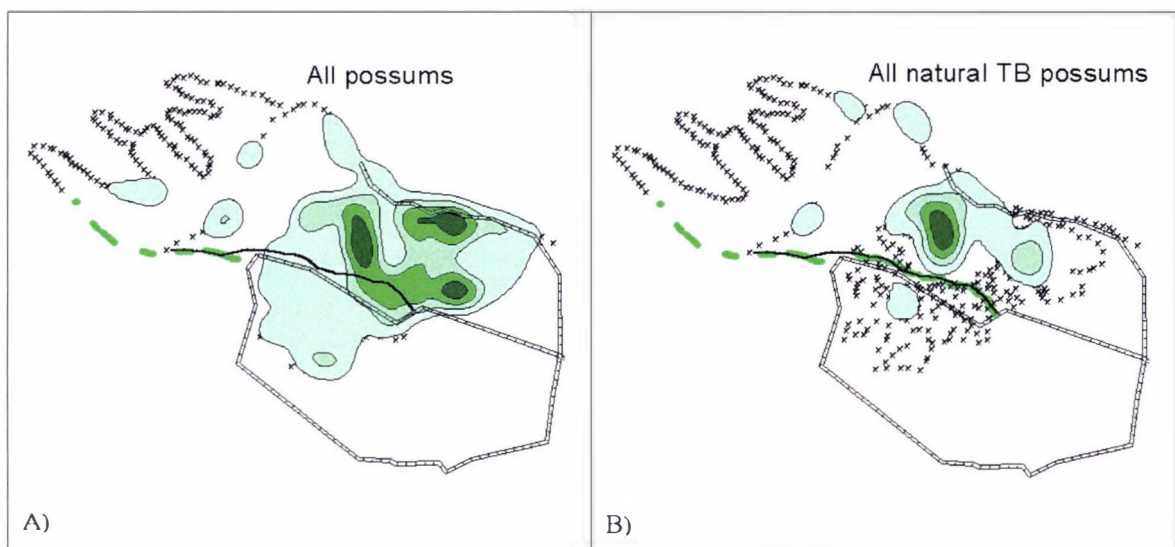


Figure 9.2

Spatial distribution of all possum captures (Figure 9.2A) and all captures of naturally infected possums (Figure 9.2B). The contour lines enclose the areas of highest capture density: inner contour line represents the highest 20%, then the outer lines enclose 40%, 60% and 80% of captures. Small crosses mark the location of traps site and lines the location of fences, tracks and creeks.

The study site was in an area with endemic possum tuberculosis. At the beginning of the study there was a pool of infected animals present on the site, a necessary condition for the study. The animals already infected were identified and excluded from the calculations of the effect of vaccination. Most were easily identified as they had been found during a previous study on the site or were found to be clinically affected the first time they were

encountered. But for those possums where tuberculosis was diagnosed during the current study and where they were on the site before the study commenced, it was more difficult to determine their classification. The duration of the preclinical phase of infection for wild possums is unknown, but may be estimated from studies in captive possums as 6 - 16 weeks (Corner unpublished observations). Therefore any possums that developed tuberculosis within 4 months of first being seen on the site were deemed to have been infected before entering the study. Only one possum fell into this category.

TABLE 9.4

The response of vaccinated and unvaccinated possums to bovine purified protein derivative tuberculin in the lymphocyte proliferation assay (bovine stimulation index).

Date	N	Treatment Group*				
		Unvaccinated		Vaccinated		
		mean	sd	N	mean	sd
July	32	1.73	1.03	26	9.23	11.41 *
Nov	13	1.52	0.74	11	5.63	3.56 *

The response of the vaccinated possums was compared to unvaccinated possums using the *t* test. * $p < 0.01$.

The prevalence of tuberculosis in the population was well estimated by regular clinical examination of the possums. Of the 308 possums involved over the 2-year study, 19 of the 24 tuberculous possums were identified by clinical examination. Repeated clinical examination compared favourably with post mortem examination as a means of detecting tuberculous in possums. Post mortem examination of 170 possums identified disease in 5 possums not showing clinical signs. Undoubtedly other infected animals went undetected, dying without developing clinical signs.

In designing the study we chose to revaccinate possums every 4 months. The average revaccination interval achieved was 5 months with possums being revaccinated up to 5 times. If vaccination is adopted for field use, populations will have to be periodically revaccinated to minimise the build up of susceptible possums. Susceptible possums will

accumulate in the population by migration of juvenile possums and breeding. The revaccination interval will probably be, for practical reasons, every 6 - 12 months.

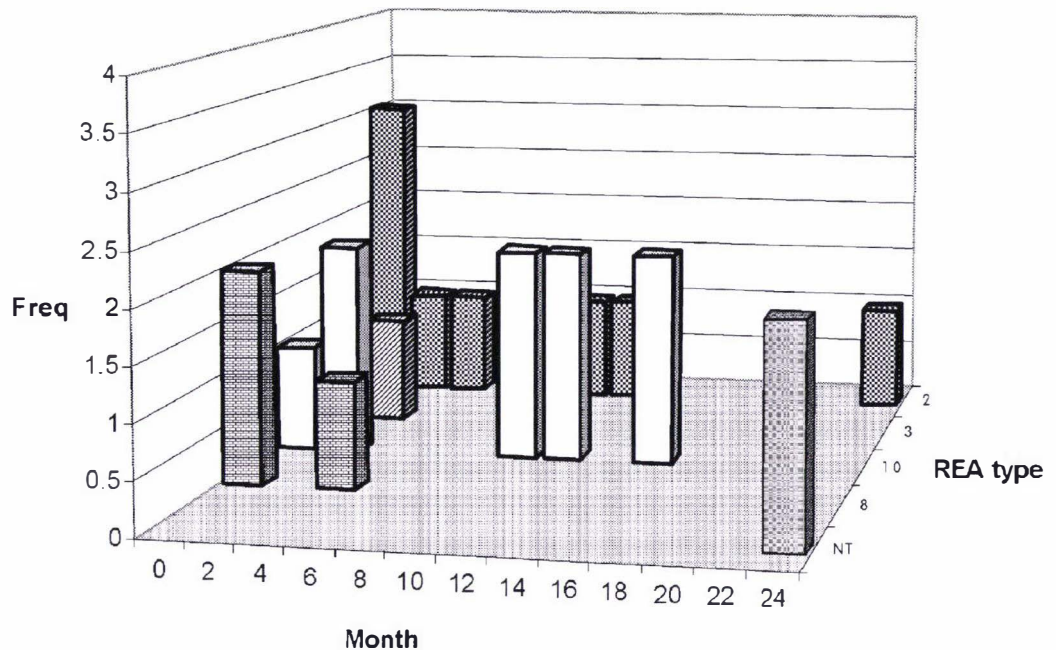


Figure 9.3

The temporal pattern of tuberculosis cases during the study of the efficacy of BCG vaccination in wild brushtail possums. Shown are the number of incident cases for each restriction endonuclease analysis (REA) type at each trapping session: type 4 (cross hatched), type 4A (diagonal check), type 4B (white), type 10A (horizontal brick) and not typed (grey).

In the allocation of possums to either the vaccinated or unvaccinated group, account was taken of where on the site they were trapped. The concern was well founded as a significant spatial clustering was observed in the study. Spatial analysis showed clustering of tuberculous possums and in the clusters cases exceeded that expected from the distribution of the population. The clustering was expected from historical data on the spatial distribution of tuberculous possums seen in previous studies at the site.

Possums were vaccinated using both intra-nasal aerosol and conjunctival instillation for several reasons. By using two different routes any failure to deliver an immunising dose by

one route would be compensated for by the other and the procedure maximised the protective response. An automated possum vaccinator, currently under development and that delivers an aerosol spray of BCG to the eyes and nose of possums, could be used to vaccinate wild possum populations.

The results of this study are very encouraging but cannot be generalised too liberally due to limitations in the design and conditions that occurred during the study. The number of incident and prevalent cases at each trapping session declined progressively during the study. No new cases were detected during the last 8 months. This may have been caused by a number of factors, including the effects of the vaccination and a steady decline in the population on the site. The overall population decreased by half due to possums being killed in the culling operation in the area around the study site, the dispersal of juvenile possums away from the study site into the depopulated area and the failure to recruit juvenile possums from the surrounding area. Vaccinating half of the population further reduced the density of susceptible possums. At the conclusion of the study the susceptible population on the site was approximately 25% of the long term mean population. At that density the transmission of infection appeared to have been reduced.

In an attempt to increase the statistical power of the study and elevate the incidence of disease in the population, possums experimentally infecting with a unique *M bovis* REA type were released onto the site. The procedure resulted in no cases due to that REA type. This was surprising considering that the infected possums survived for 8 to 21 weeks, all developed extensive lung disease and all were excreting *M bovis* from the respiratory tract. The transmission of infection between captive possums held in small cages or communally in large pens has been difficult to establish experimentally (Corner unpublished observations). Transmission rates were substantially increased in these studies when social interactions and social ranking of the possums were included in the study design. For transmission to occur in the wild, in addition to a source of infection, there must be interactions between possums of a suitable nature and duration for the infectious aerosol to be transmitted.

The demonstration that BCG vaccination induced a significant level of protection against naturally transmitted infection validates previous vaccination studies in captive possums (Buddle et al 2001; Corner et al 2001). The captive possum studies have shown that vaccination has a protective against experimental challenge, but all possums,

vaccinated or unvaccinated, developed fulminant, rapidly fatal disease. The presence of protective immunity was shown by a difference in the severity of the disease when vaccinated and unvaccinated possums were compared. This field trial showed that BCG vaccination prevented the establishment of infection and disease. The justification for conducting a field vaccination trial was the demonstration of protection in captive possum studies. Those studies enabled different vaccination procedures and vaccines to be compared and they are simpler, faster and more easily conducted than field trials. Now, against the yardstick of the protective immunity shown in this field study, further research into improved vaccines and vaccination methodology can be conducted with confidence. The results of this study are applicable to the delivery of live BCG vaccine by other routes. If a suitable means of encapsulating BCG to enable oral delivery is developed, the results of this study will enable rapid utilisation of that technology.

Vaccination offers an alternative or complementary strategy for the control of tuberculosis in possums. Vaccination could be used at two levels, as part of on-farm control programs where diseased possums are the source of infection for livestock, and at a district level to control disease over larger areas and prevent the expansion of areas infested with infected possum populations. Current strategies have failed to contain infected populations. New control strategies incorporating vaccination could lead to a reduction in the size of infected areas and also a reduction in the use of toxins. The implementation of such strategies could be used to allay the growing public concerns over adverse environmental effects of toxins and animal welfare concerns over the continued use of toxins in possum control.

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SECTION E

Conclusion

Chapter 10

General Discussion

Objective of the Research

The objective of the research was to better understand the potential of BCG as a tuberculosis vaccine for possums. With the results we planned to develop a strategy for the utilisation of BCG in wild possum populations. When the project commenced there had been substantial investment in tuberculosis vaccine research but the benefits would only be gained if the vaccine was effective and could be delivered to wild possums. Before vaccination could be adopted as a strategy for controlling tuberculosis, a number of questions needed to be addressed.

Vaccination Studies

When this research project commenced only three papers had been published on the protective effect of BCG vaccine in possums, all from the same research group (Aldwell et al., 1995a, 1995b; Buddle et al., 1997). The only feasible delivery route that had been shown to offer a protective effect and that could be adapted for field use was intranasal aerosol. However, before aerosol delivery could be considered for field use, it had to be shown to be a robust procedure and that the protection induced would last sufficiently long to protect possums before revaccination of a population was required. When these questions had been answered, we could examine how to adopt BCG as a tool for the control of tuberculosis in wild possums. Therefore the initial experiments focused on establishing that BCG would induce a protective response and also examined the duration of protection.

The first experiment confirmed that intranasal aerosol vaccination was effective and that protection following a single vaccination was still evident after 12 months, although the level was waning. In the context of field use it is difficult to assign significance to the residual level of protection that was observed after 12 months. The experimental challenge procedure used was severe and all possums succumbed to the challenge. Under field conditions, the residual level of protection may be sufficient to prevent infection.

Having established that aerosol vaccination would protect for an acceptable duration, the emphasis shifted to the means of delivering vaccine to wild possums. It was essential that vaccine delivery be accomplished cheaply and efficiently. If a device was used to dispense the vaccine, it had to be simple in design and operation. Even though repeated

vaccination of humans has no adverse effects on the protection induced by the first vaccination (Cohn, 1997), we undertook an investigation of the effects of repeated vaccination on protection. If it were necessary to prevent possums from repeatedly accessing vaccine, a more costly and complicated device would be needed. The study on repeated vaccination demonstrated that repeated vaccination in fact enhanced protection.

Of the three vaccination regimes in the study, 12 weekly doses induced the greatest protective effect, but there were no consistent differences between two doses given 6 weeks apart and a single dose, in any of the measures of protection used. These findings are at odds with those of Griffin et al. (1999) who demonstrated increased protection against disease, but not against infection, in deer given two doses of BCG two months apart. Differences in the route of administration, dose rate and host species may account for these differences.

A surprising outcome of the multiple vaccination experiment was the absence of any demonstrable boosting of cell mediated immunity (as measured by the lymphocyte proliferation assay) following revaccination. Revaccination in humans does not increase the intensity of the immune response, nor does it extend the duration of protection (Cohn, 1997). We only looked at boosting after 6 weeks and there is good reason to examine the effect of revaccination at 6 to 12 months. Populations will need to be revaccinated to maintain a low level of susceptible possums. For a number of conceivable reasons some possums may not be exposed to revaccination for a long time. However, irrespective of any specific need to boost the immune response in individual possums, re-vaccination of individuals may occur as a consequence of repeated vaccination of populations.

Because aerosol delivery from a small handheld dispenser was effective, we set out to design a device that would deliver to possums a vaccine aerosol. In developing the concept of a possum activated self-vaccinator, severe design limitations were imposed when the target for the aerosol spray was restricted to the area of the external nares. By broadening the target area to include the eyes, the performance criterion could be relaxed. Although conjunctival vaccination had been used to vaccinate poultry against Newcastle disease (Bell et al., 1995) and sheep and goats against brucellosis using live *Brucella melitensis* strain Rev 1 (Blasco, 1995), the efficacy of conjunctival vaccination of possums with BCG, or any other animal species for that matter, was unknown. A study of conjunctival vaccination showed this route to be as effective as intranasal aerosol.

In the captive possum experiments the animals were housed communally in large outdoor wire-mesh-covered enclosures after the manner of described by McLeod et al. (1997). It had been reported that captive possum suffered from immunosuppression as a result of the stresses of capture and captivity (Buddle et al., 1992). By employing communal housing in large outdoor pens, we hoped to decrease the stresses associated with captivity and approximate conditions in the field. No specific benefits were seen when our results were compared with previous findings where possums were housed singly. Communal housing did however lead to considerable savings in the cost of animal accommodation, and the capacity to conduct studies on a much larger scale.

Experimental Infection and Transmission Studies

The intratracheal challenge procedure, used in the first experiments, was not entirely suitable for our studies. Although it provided an assured level of exposure and repeatable results (Pfeffer et al., 1994), all infected possums developed fulminant, rapidly progressive disease irrespective of the vaccination regime used. All infected possums had extensive lung lesions but few developed the pattern of clinical signs that were recorded in studies of wild naturally infected possums (e.g. Jackson et al., 1995). After experimental challenge, possums died in 6 to 8 weeks, with a clinical phase of 1 to 2 weeks. This disease course is considerable faster than seen in wild possums. In wild possums there is a preclinical phase of unknown duration and the median survival time after the development of clinical signs we found to be 2 months (Pfeiffer, 1994; Jackson, 1995) - and Jackson (1995) found one possum lived for more than a year. The question then arises as to the validity of the results of vaccine studies using this challenge procedure when the possums died so quickly. The experimental vaccination and challenge studies showed that the vaccine could alter the nature of the disease after challenge. But the severe nature and rapid development of infection would not enable the detection of subtle differences between vaccines or vaccination regimes. In an attempt to refine the vaccination and challenge paradigm two alternative methods of challenge were examined; natural transmission between experimentally infected possums and susceptible in-contact possums, and the conjunctival route of infection.

Designing a successful natural disease transmission study proved to be more involved than was originally thought. Transmission of tuberculosis between possums in the same small cage and between possums in the large pens proved difficult to establish. When only

the proximity of infected and susceptible possums was considered, either no transmission occurred or the rate was low. Other researchers have reported similar difficulties in reliably establishing transmission between captive possums (O'Hara et al., 1976; Corner and Presidente, 1980, 1981). When we were unable to demonstrate transmission in a colony comprising 19 experimentally infected possums and 3 susceptible in-contact possums, we concluded that simple random allocation of possums to treatment groups was not appropriate.

Tuberculosis is transmitted between possums principally by infectious aerosols (Jackson et al., 1995b). Therefore, the risk of disease transmission is a function of both distance between animals and the duration of the interaction. After observing the behaviour of possums in our colonies it was apparent that transmission was most likely to occur when possums slept together in the same den, sharing a common airspace. Therefore den sharing could be used as a surrogate measure for the frequency and duration of social interactions. Using observations on den sharing a model of the social organisation of the communally housed possums was developed. Social network analysis concepts, structures and statistical procedures were used to develop the model.

When we selected possums to experimentally infect based on social behaviour, the rate of transmission was significantly increased. The possums that became infected were more socially active than those that remained free of infection. No effect of vaccination was found under these conditions because of the confounding effect of social position on the risk of an individual becoming infected. That is, we could not control the transmission risk well enough to use natural transmission in place of intratracheal infection as the routine method of challenge.

The other infection procedure, conjunctival infection, was shown to be useful for infecting possums. We were able to reliably establish infection by this means and the disease process had many of the cardinal features of the natural disease in wild possums, features not seen in possums infected by intratracheal inoculation. After inoculation, the infection progressed slowly, with possums surviving in excess of 4 months. Most possums developed palpable lesions in superficial lymph nodes and they also had pulmonary lesions. It will now be necessary to determine if conjunctival infection can provide a more sensitive evaluation of vaccine efficacy than intratracheal infection.

Although natural transmission was found unsuitable for challenging possums in vaccination studies, it may be suitable for studying some aspects of the pathogenesis. The study of pseudo-vertical transmission and the efficacy of post-infection vaccination are two situations where this may be useful. As observed above, intratracheal infection leads to a fulminant, rapidly fatal disease. After natural transmission of infection, the disease progressed slowly, the levels of cell mediated immune responses seen, covered a wide range and the pattern of lesions mimicked that seen in naturally infected possums. For the study of pseudo-vertical transmission a long period of infectiousness is required to allow the birth and development of the pouch young. Both conjunctival infection and natural transmission resulted in slowly developing infection that would allow for pseudo-vertical transmission. It is not possible to investigate post-exposure vaccination using intratracheal infection because of the fulminant nature of experimental infections. Conjunctival infection may allow sufficient time for any protective immunity induced by post-exposure vaccination to be demonstrated. Natural transmission would be unsuitable for such studies due to the difficulty of controlling for the effects of social behaviour on the risk of disease transmission.

Field Studies

Having successfully demonstrated the efficacy of BCG vaccine in captive possums and removed some of the perceived problems of vaccinating wild possums, the focus of the research changed to address vaccine use in wild possum populations. The endemically infected population at Castlepoint was used, but before the vaccine study could be conducted we first had to establish the pattern of disease in the population. The first study commenced immediately after possums were eradicated from the site. It examined the temporal and spatial nature of tuberculosis as it re-established in the regenerating population.

Tuberculosis reappeared on the study site soon after depopulation. Within 4 months of depopulation tuberculous possums were found on the site. Within 8 months the population had returned to 50% of the 5-year pre-eradication average. The early cases, found within the first 12 months, were in mature possums that had extended their range into the depopulated area and in a young possum that probably entered the site during juvenile dispersal. The first cases in resident possums were not seen until the second year. Re-emergence of tuberculosis on the site was due to immigration of infected possums, and not

due to survival of *M. bovis* in the environment. In other areas, where culling programs are less than 100% effective, some diseased possums may survive to re-establish infection but environmental survival of the bacteria is unlikely to play more than a minor role in the persistence of infection.

Restriction endonuclease analysis (REA) of the *M. bovis* strains in diseased possums demonstrated the dynamic nature of tuberculosis in the possum population. Four different REA types were identified in the population during the 3-year study. Each type showed a different temporal and spatial pattern. Each established a focus of infection, each was repeatedly reintroduced and all but one type persisted on the site for the duration of the study. At the end of the study, when there was an upsurge in the incidence of diseased possums, the three types present showed a similar pattern of increased incidence. The upsurge in disease incidence at that time may have occurred for several reasons. It followed soon after the population peaked and the increased density may have facilitated increased transmission. Co-incident with the upsurge in the study population we saw an increase in the number of cases in immigrant possums. By this we reasoned that whatever triggered the increased incidence on the study site may have also influenced disease prevalence in the surrounding area.

Evaluation of BCG vaccine in the wild possum population showed it had high efficacy. There were significantly more cases of tuberculosis in unvaccinated possums than in vaccinates, with a relative risk of tuberculosis in unvaccinated possums of 3.21. The vaccine efficacy was 69%. Over 300 possums were recruited to the study and approximately 50% were vaccinated, using both intranasal aerosol and conjunctival instillation. They were revaccinated on average every 5 months, some being revaccinated 5 times.

A combination of intranasal aerosol and conjunctival vaccination was used to ensure that each possum was vaccinated effectively. The possums were handheld when vaccinated which provided a high level of control; we knew which possums had been vaccinated and when. Such a level of control is essential in early field evaluation trials.

The study was designed to test vaccination efficacy and not to test the vaccine delivery system. Aerosol vaccination is, however, the only delivery system that has been proved successful in captive possum studies, and it is suitable for use in a possum-activated

vaccinator. An aerosol can, charged with a suspension of BCG, could be incorporated into a device where activation of the spray would be triggered by a possum encouraged to enter the vaccinator in response to its novelty and possibly in response to a chemical lure.

The efficacy achieved in this trial should be applicable to vaccine delivered in an oral bait, should an effective oral delivery system be developed. Currently there is no procedure for oral vaccination, hence aerosol and conjunctival vaccination were used in the field study. The current results should enable oral vaccination to be adopted quickly. Both oral and aerosol vaccination may have different characteristics when used in the field and may be used under different circumstances; they could be used concurrently. If a vaccine superior to BCG becomes available these results should ensure rapid development and release.

Even though a significant effect of vaccination was demonstrated, there were some minor aspects of the study that limit the extent to which the results can be generalised. There was a steady decline in the population on the site during the two years of the study that would have decreased the risk of disease transmission. At the end of the study the population was less than half the long-term mean. As part of the national control program, the area around the site was subjected to a culling program that coincided with the period of the study. The decline in the study population was caused by natural death, plus additional possums being killed on the periphery of the site, juveniles lost due to dispersion, and, because there were no possums in the surrounding area, a decrease in immigration of juveniles. If vaccination were to be utilised as a disease control tool it will probably be employed in populations regenerating after culling and later when these populations stabilise in number.

The second limitation was the low proportion of the population that were vaccinated (50%). If vaccination is used in the field, the intention will be to vaccinate a higher proportion of the population. Modelling predicts that a vaccine that falls well short of 100% efficacy could be used successfully if a sufficient proportion of the population were protected (Roberts, 1996). Vaccination of a proportion of the population would decrease the probability of contact between diseased and susceptible possums, the so-called "herd immunity" effect (Fox et al., 1971). Modelling could be used to determine the optimum revaccination frequency based on estimates of the rate of recruitment of susceptible

possums. More data from field studies are needed to develop new models or modify existing ones and to test their predictions.

The most important question relating to BCG use, and not so far addressed, is the ability to control tuberculosis in possum populations. Although the method of delivery has still to be finalised, information on the ability of vaccination to control disease is necessary to justify continued vaccine research. A controlled field trial is needed to show that vaccination can significantly affect the incidence and prevalence of tuberculosis where all possums in the population are vaccinated. Such a study should be conducted concurrently with further development of both aerosol and oral delivery systems so that implementation of vaccination can be achieved at the earliest possible time.

Epidemiology

Two aspects of the pathogenesis of tuberculosis in possums were clarified during the experimental and natural transmission studies; the duration of preclinical infection and the pre-eminence of the aerosol route in naturally transmitted tuberculosis. Some aspects of the pathogenesis of tuberculosis in possums can only be determined from controlled studies in captive possums. Longitudinal studies by Pfeiffer (1994), Jackson (1995) and the current study, have established the mean survival time for possums, once they had developed clinical disease, as 2 months. The duration of preclinical disease cannot be determined accurately by field observations because the point of exposure is always unknown. Based on bacteriological finding and the develop of lesions, Jackson et al. (1995d) estimated the duration of the preclinical stage (no macroscopic lesions) plus early clinical (few macroscopic lesions) stage to be several months to years. In the conjunctival infection study, the minimum preclinical/early clinical period was 6 - 9 weeks and was influenced by the infecting dose, being shorter with higher doses. In the natural transmission study the preclinical/early clinical period for all infected possums was 16 - 20 weeks. The preclinical/early clinical period in wild possums therefore was well estimated at the lower end by Jackson et al. (1995d).

The primary route of transmission between possums was reconfirmed as respiratory. Of the possums infected by natural transmission only one of the 17 did not have lung lesions. The one exception had a lesion in a parotid lymph node. The presence of a single lesion in a head lymph node could be the result of infection established in tissues drained by the

node, that is, the mucous membranes of the nasal and buccal cavity. But this is not necessarily true. Although an attractive explanation, it is fallacious to presume that the location of a single lesion indicates the route of infection. After conjunctival infection the only lesions present in one possum were in the inguinal lymph nodes, far from the primary site of infection. Infection of the inguinal and axillary lymph nodes is a typical feature of tuberculosis in possums. After initiation of infection *M. bovis* infection rapidly generalises and most possums develop pulmonary lesions, with many developing lesions in a range of other tissues (Cooke et al., 1999).

As described above, it was surprisingly difficult to establish transmission of infection in a variety of pen conditions and in the field. Generally tuberculous possums cannot be considered highly infectious. In studies where susceptible possums were housed with possums that were dying of tuberculosis and had extensive pulmonary lesions and large numbers of bacilli in tracheal exudates, there was limited or no transmission. In the repopulation study, after the first 4 infected possums were detected there were no new cases with some REA types for 11 - 31 months. Similarly, during the field vaccination trial, when 8 experimentally infected possums were released on to the site, no transmission occurred, despite the experimentally infected possums living in areas of high possum density. Factors other than proximity influence the risk of transmission, as was demonstrated in pen studies. Social behaviour was used to help explain the pattern of transmission.

That additional factors are involved in transmission of tuberculosis has been established in other species. In cattle and badgers it has been shown that they do not become infectious until the disease has become well advanced and there are large numbers of bacteria being excreted (Nolan and Wilesmith, 1994; O'Reilly and Daborn, 1995). In humans infected with tuberculosis it appears that only certain individuals become infectious, as household contacts that included marital partners of smear-positive patients, frequently did not become infected (O'Reilly and Daborn, 1995). In these three hosts it was found that infectiousness was directly related to the number of bacilli being excreted, the route of infection, the infective dose, the period of communicability (duration of exposure), and host susceptibility (O'Reilly and Daborn, 1995).

Future Research On Vaccine Delivery

As a means of delivering BCG vaccine to wild possums, aerosol vaccination has great appeal. It has been proven to be reliable and repeatable, and is a simple procedure in both captive and wild possums. Both sedated and fully conscious possums have been vaccinated using small handheld atomisers. Aerosol vaccination could be easily adapted for use with wild possums, using a device based conceptually on a bait station. A spray of live BCG suspension would be directed at the possum's nose and eyes from a pressurised aerosol can. By this means BCG would be delivered to the mucous membranes of the upper and lower respiratory tract. In humans, vaccination via the respiratory route has been identified as deserving of research (Gheorghui, 1990). Very few live BCG are required to initiate infection on a mucous surface and therefore to induce immunity (Middlebrook 1961). The live bacterial component of the vaccine is likely to be the cheapest part in the manufacture and delivery process. Therefore it should be possible, on financial and manufacturing grounds, to deliver a dose far in excess of that needed to produce a protective response. This would allow for a large margin for error, and so aerosol vaccination would provide a robust delivery system.

Possums are naturally inquisitive and will investigate novel objects in their environment (Norton, 2001). This behaviour has been harnessed in the development of a prototype vaccinator, with the attractiveness of the device being enhanced by the inclusion of a chemical lure. The vaccinator employs the possum's weight and movements to release an aerosol spray. The prototype has been developed using aerosol cans containing a dye in place of the vaccine. Research is now required on the development of the vaccine spray and refinement of the vaccinator before commercial production can progress.

The vehicle in which the BCG will be suspended in the aerosol can is the key component still requiring research. The vehicle must be a sprayable liquid that maintains BCG in a viable state, in high concentration, across a wide range of temperatures and for sufficient time to allow for a cost-effective period of storage and use. Once the vehicle is formulated there will be a period of trialing in the laboratory to establish shelf life, then with captive possums to establish immunogenicity, and in the field to establish operational efficacy.

Development of an oral delivery system for BCG is not as well advanced as the aerosol system. The early attempts at oral delivery failed to invoke a protective response, presumably due to the destruction of BCG by gastric secretions (Aldwell et al., 1995a). When BCG was delivered directly into the duodenum, protective immunity similar to that following intranasal aerosol was achieved (Buddle et al., 1997). If the bacteria can be protected from gastric secretions, an oral bait vaccine is feasible. Development of strategies for using oral bait vaccine should proceed rapidly as researchers can draw on the considerable knowledge acquired through research on toxin baits. Unlike aerosol BCG, vaccine baits will probably have a high unit cost due to wastage from loss of bacterial viability, excessive consumption by possums and baits taken by non-target species. These will be problems unique to that delivery system. However, neither the aerosol nor the oral delivery system is currently at a stage of development where a decision is needed as to whether one or both should be pursued to the point of commercialisation.

Field studies in the immediate future will have to use aerosol and conjunctival vaccination. Vaccination of hand held possums will be used to enable strict control of vaccination, to ensure effective vaccination and to determine which possums have been vaccinated, when and how often. This does not mean that aerosol vaccination is the best delivery system, just the best tool available for the next field study.

Another unanswered question relating to the future use of BCG vaccine is that of dissemination of BCG from one possum to another. Exposure to live BCG results in infection on the exposed mucous membranes, which then extends to the lymphoid tissue draining the membrane. BCG does not persist on mucous membranes, so dissemination to other possums is unlikely, other than by mechanical means following aerosol vaccination. As possums are solitary, this is unlikely except between a mother and her pouch young. Aerosol vaccination may result in the pouch young becoming protected as a result of exposure to the vaccine spray, or indirectly from contact with the dam while she has BCG on her fur. Oral baits are unlikely to lead to protection of pouch young until the young start ingesting solid food. So for effective vaccination of a population by the oral route each individual would have to be exposed directly to the vaccine source.

Control Strategies Incorporating Vaccination

With the efficacy of vaccination established, issues relating to strategies for vaccine use in the field can be addressed. Modelling has been a valuable tool in guiding and evaluating the research on tuberculosis in New Zealand, and in the potential use of vaccination (Barlow, 1991; Roberts, 1996). Vaccination will probably be used after an initial cull, as models that retain continued killing together with vaccination do not show any advantages over killing alone (Barlow, 2000). However, the predictions of models are only as good as the quality of the field data they are based on. With the new data on vaccine efficacy the available models can be refined. They should then be used to explore ways to optimise vaccine use, to address the question of population coverage and the frequency of revaccination.

Strategies for vaccine use will have to ensure direct exposure of as many individuals in the population as possible. With aerosol vaccinators, their distribution will be dictated by the home range of possums and frequency with which possums cover their range. To have a high probability of successfully vaccinating every possum, a vaccinator will have to be placed within the home range of every possum. The size of a home range is habitat dependent but typically ranges from 1 - 4 ha, with ranges of individuals overlapping extensively (Cowan and Clout, 2000). Possums tend to cover the area of their range every 3 - 5 days. Vaccinators could be left in place for this period then moved to another area. By moving vaccinators regularly large areas may be covered with the minimum number of vaccinators. Similarly, the frequency of revaccination of a population will influence the number of vaccinators required. Wild populations will require revaccination to minimise the proportion of susceptible possums in the population. Susceptible possums will be those missed at each vaccination, unvaccinated immigrants and recently born animals. The optimal interval for revaccination would probably be 3 - 6 months. Revaccination may also boost waning immunity and so maintain a high level of protection in previously vaccinated possums. The frequency of revaccination will be driven, therefore not by immunological consideration, but factors relating to population dynamics, benefit-cost analysis and pragmatic decision-making.

We should not be prejudiced against BCG by the controversy in human medicine. BCG has many characteristics that make it the "ideal" wild animal vaccine. It is effective, easy to grow, cheap to produce, easy to handle (Buddle et al., 2000; Skinner et al., 2001), and is

the safest human vaccine produced (Hanson et al., 1995). In vaccinating wild possums the primary aim is to reduce the rate of transmission, the incidence and prevalence of disease, and transmission from possums to other species. A vaccine need not be 100% effective and the entire population need not be protected. The effectiveness of a vaccination program will result from both direct and indirect benefits to the single individual as well as the total and average benefits to the entire population (Haber et al., 1991). The establishment of herd immunity is a real and valuable feature of vaccinating populations and this can be achieved with BCG. At present BCG is all that is available and its effectiveness in possums has been shown.

The majority of the research findings with aerosol and conjunctival vaccination will be directly applicable to future developments in vaccines. If a vaccine with better performance characteristics is developed or a better delivery method becomes available, the results of current research will allow for a more rapid evaluation and introduction of the technology. In the immediate to long term, research and field use of tuberculosis vaccines in wildlife will rely on BCG. A new animal vaccine could be a spin-off from the research into human tuberculosis vaccines. However, there is no need to wait for a new human vaccine superior to BCG as BCG has been shown to be effective. In addition, progress towards a new human vaccine has not been very fruitful. Dr D McMurray (pers comm. 2000) from the tuberculosis vaccine testing centre at Texas A&M University, has tested over 70 candidate vaccines but none was more effective than BCG. BCG is currently the only vaccine available for use in humans and will be the benchmark for new vaccines.

A new human vaccine may not necessarily have the characteristics that will make it a good vaccine for animal use, or more especially, for use in wild possums. The ideal human vaccine may have different characteristics from an effective animal vaccine. The former should protect better than BCG, provide long lived, if not life long, immunity in long-lived species, protect against primary infection and endogenous reactivation, and immunised subjects should be distinguishable from infected patients (Gheorghiu, 1990; Bloom and Fine, 1994; Lowrie et al., 1995). For animal use some of these characteristics would be an advantage, but for wild animals protection need only last for a few years (production life or total life span) and protection must be achieved with a single dose delivered by non-invasive means. If a new vaccine was developed for use in domestic or wild animals, obtaining approval for its use may take many years of administrative procedures.

There are two basic approaches for using a vaccine to control tuberculosis in wild possums. Firstly, for control at the individual farm level and secondly at a regional level. These can be evaluated by field trials and modelling. At the farm level, where possums are a proven source of recurrent problems in livestock, vaccination could be added to the on-farm disease control program. At the regional level, vaccination would be used to contain infected possum populations within known vector risk areas and possibly used to reduce the size of these areas. Although the current control program has been successful at reducing the number of infected herds and livestock reacting to the diagnostic tests, it has been much less successful at preventing the expansion of existing vector risk areas or the establishment of new vector risk areas (AHB 2000b). Immune buffer zones may be more effective at containing diseased possum populations.

In the use of vaccination on farms and in regions, culling of possums using conventional methods would typically precede the application of vaccine. The culling would decrease the pool of infected animals and decrease the risk of infection moving to adjoining possum populations and domestic livestock. Under normal culling programs some possums survive and it is the tuberculous survivors and infected immigrants that cause the recrudescence of infection in the recovery phase of the repopulation. If transmission from these animals to disease-free survivors, disease-free immigrants and possums born in the area could be neutralised by vaccination, the disease may remain at low prevalence and not be a risk to livestock. The disease may even die out.

Programs aimed at containing the outward expansion of tuberculosis from known infected possum populations that are based on culling, have not been successful (AHB, 2000b). Success has been achieved (possibly) in only a few small areas, whereas the overall area of land where tuberculous possum populations are found has expanded. Vaccination could be used to effectively contain infected populations. A buffer zone of immunised possums would provide a barrier where the effect of diseased animals would be neutralised. There would be a decreased probability of infected possums settling because the area would have an established population. An immune buffer zone would be superior to a culled buffer zone as it would remove the effect of a low-density population on survival of dispersing juveniles. The low densities would result in higher settlement probability and higher survival probability within the culled area. In time the areas with

disease populations inside the immune buffer zone could be included to roll back the disease front.

BCG could be used for an additional purpose, as a live bacterial vector for delivering other antigens to possums (Hanson et al., 1995). If immunocontraceptive vaccines are developed for the control of reproduction in possums, BCG could be used as the vector. As a live vector it would persist for days to weeks in the host continually secreting antigen (Uren et al., 2000). A strong immune response would also be ensured because mycobacteria have mycolic acid in their cell wall, a potent immunological adjuvant that enhances the immunological response to excreted antigens (Barletta et al., 1990). The use of a live vector would avoid the expensive down stream processing required with a purified recombinant antigen (Moore, 2000). When used in this manner both control of reproduction and of tuberculosis could be achieved at the same time.

Just as there are risks with the persistence of tuberculosis in wild possums and domestic livestock, the use of BCG in animals has its own risks. There are few direct risks to humans when handling BCG vaccine, as it is the safest of all human vaccines (Hanson et al., 1995). The main risk to humans is an indirect economic risk from the accidental exposure of livestock. Vaccinated animals may give a positive skin test response (Buddle et al., 1995b) and if it were misinterpreted as evidence of infection with *M. bovis*, the animal would be condemned and the owner penalised.

Part of the original justification for a national bovine tuberculosis control program was the concern for human health. The risks came primarily from contamination of cows' milk with *M. bovis*. Before pasteurisation of milk was introduced the majority of childhood tuberculosis was due to *M. bovis*. Currently in countries with high standards of animal husbandry and dairy production, bovine tuberculosis accounts for a very small proportion of human tuberculosis cases. *M. bovis* infection remains contained and subclinical in most people. The risk of developing active disease increases with age and in patients who are immunocompromised. In a case-control study in Tanzania, people infected with human immunodeficiency virus (HIV) were found to be 8.3 times more likely to develop tuberculosis than those not so infected and 29% of tuberculosis cases occurred as a consequence of HIV infection (van der Broek et al., 1993). "It has become clear that, worldwide, tuberculosis is the most common opportunistic infection occurring in patients with AIDS" (Smith and Moss, 1994). Infection with *M. bovis* also occurs and has the same

consequences (WHO, 1994). Therefore there is need to control bovine tuberculosis both in domestic animals and wild animals where HIV is prevalent. Vaccination of livestock may be the preferred option in underdeveloped countries where a test and slaughter program cannot be afforded.

The results of these studies on BCG vaccination of possums against tuberculosis will be applicable to the use of BCG in other species of wild animal. The course the research followed for possum vaccination could be used to design a research program for other species. Aerosol vaccination should theoretically be effective in all animal species and a species-specific vaccinator could be designed to deliver the vaccine. The delivery of oral vaccine to other species, if an effective encapsulation procedure is developed, is more problematic due to differences in diet, digestive processes and behaviour. The groundwork for the use of BCG vaccination in wild animals has been established and now requires research to address the specific problems of delivery and effectiveness in the animal populations affected.

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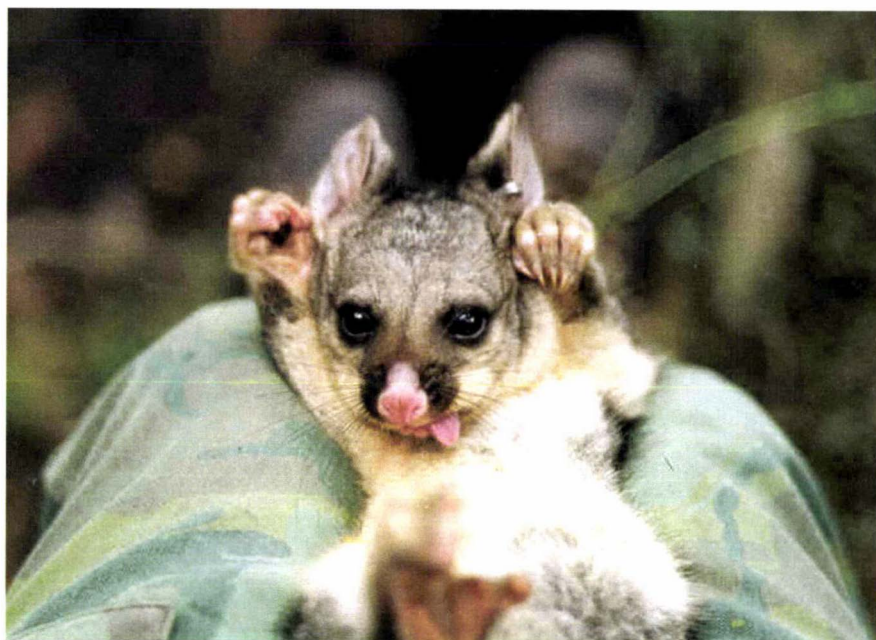
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