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Newly attenuated *Mycobacterium bovis* mutants as vaccines against bovine tuberculosis, particularly for possums

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ABSTRACT

Bovine tuberculosis costs New Zealand more than $80 million per year, mostly because extensive areas of the country are occupied by brushtail possums infected with *Mycobacterium bovis*. AgResearch has a major programme to produce new live tuberculosis vaccines that can be delivered to possums. Primary work involved development of molecular biological methods to enable genetic manipulation of *M. bovis*, including the production of random and specific mutants. Many avirulent mutants of *M. bovis* have been produced and their vaccine efficacy has been compared to BCG in guinea pigs. Selected mutants that perform at least as well as BCG are retested in guinea pigs using an extended vaccination protocol in which animals are pre-sensitized to environmental mycobacteria to mimic natural exposure. Ten candidate vaccines that have induced good protection in guinea pigs have been subsequently tested as vaccines in possums. While the protective efficacy of an *M. bovis* mutant inoculated into guinea pigs reliably indicated that some protection would be induced in possums, the most protective mutant in guinea pigs was different from that in possums. This illustrates the importance of testing in the target species as part of new vaccine development. An important outcome of this work was the identification of an operon in *M. bovis* whose inactivation produced an avirulent *M. bovis* vaccine candidate that was better than BCG in protecting possums from experimental tuberculosis. Allelic exchange methods are now being used to produce vaccine strains with multiple specific mutations to improve safety and immunological characteristics.
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1. Introduction

Eradication of bovine tuberculosis in New Zealand from all infected wildlife, particularly Australian brushtail possums (*Trichosurus vulpecula*), is a daunting challenge. The current strategy for bovine tuberculosis which relies heavily on large-scale poisoning of possums is successfully achieving reduction of the level of infection in farmed animals and in some selected wildlife populations (Ryan et al., 2006). However, it will cost the country more than $1 billion over the next 15 years if current expenditure is maintained, and even in that time is not expected to achieve eradication of infected wildlife from more than a quarter of the area it now occupies. Vaccination is the only new alternative control method that can be implemented in the next 10 years. The current tuberculosis vaccine, BCG, was developed more than 80 years ago and is an avirulent live strain of the bovine tuberculosis organism, *Mycobacterium bovis*. Attenuating mutations in BCG have been identified (Collins et al., 2003; Guinn et al., 2004; Brosch et al., 2007), but the mechanisms by which these mutations interfere with virulence of *M. bovis* and enable BCG to induce protection are still poorly understood. Although BCG is the world's most widely used human

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vaccine and has been tested in possums, cattle and many other animals (Buddle et al., 2006), protection has been highly variable. While some studies have reported good protection (Fine, 1995; Tompkins et al., 2009), other studies have found only modest or even no protection and it has been concluded that the efficacy of BCG has generally been disappointing in the developing world (Andersen and Doherty, 2005). A recent field trial using orally administered BCG in possums gave encouraging results (Tompkins et al., 2009), indicating that a better vaccine than BCG might achieve a dramatic reduction in wildlife transmission. Many unsuccessful attempts have been made to develop non-living sub-unit vaccines that are better than BCG (Orme et al., 2001; Orme, 2005) and it appears that BCG being a live organism is important for inducing protection. The convergence of advances in molecular biology with the inability to eradicate tuberculosis from human populations has led to many different types of tuberculosis vaccines being developed for human use (Orme et al., 2001; Orme, 2005). Much of this work is focused on non-living vaccines because of anticipated easier regulatory approval for human use. To be effective, these vaccines require multiple inoculations at timed intervals and will most probably need to be administered by injection. None of these requirements is suitable for a wildlife vaccine. In contrast, live avirulent strains can be given once and can be administered orally, both features that suit vaccine delivery by oral baits (Cross et al., 2007). This paper reports the strategy being used to produce a new live tuberculosis vaccine for possums.

2. Strategy to produce a new live *M. bovis* vaccine

The overall strategy being used involves several disciplines and many different steps. The first step was to develop methods of mutagenesis in *M. bovis* in order to produce large numbers of random mutants by illegitimate recombination and transposon mutagenesis. These mutants were subjected to a range of in vitro tests that were judged likely to select those that had a raised likelihood of being avirulent. These selected mutants were tested for virulence in guinea pigs. The vaccine efficacy of avirulent mutants was then assessed in guinea pigs using a low dose aerosol challenge with a New Zealand *M. bovis* strain. Some mutants that gave comparable protection to BCG in guinea pigs were then retested in a second guinea pig model in which the animals were first sensitized by the oral administration of a local strain of *Mycobacterium avium* subsp. *avium* to mimic the sensitization to environmental mycobacteria that often occurs in wildlife. Mutants that performed at least as well as BCG in guinea pigs were tested for virulence in possums and then for their vaccine efficacy in possums. Random mutants that showed promising vaccine characteristics were reconstructed using allelic exchange methods to delete the appropriate gene(s) without incorporating any transgenic antibiotic resistance genes in the final product. These mutants were then retested in animals. The best vaccine candidates are being characterized by a range of methods, and attenuating and immune-modifying mutations are being combined in single strains. These strains with multiple mutations also require testing in animals as this iterative process advances towards the final vaccine product. It is expected that this vaccine will contain no foreign DNA and will have at least two independent attenuating mutations. The vaccine is also likely to be constructed with a mutation in a gene such as *secA2* whose inactivation leads to modification of the host's immune system (Hinchey et al., 2007) and a mutation in a gene such as *esxA* (the ESAT6 gene) that will enable vaccinated animals to be distinguished from naturally infected animals (Wards et al., 2000). The general iterative process being followed is outlined in Fig. 1.

3. Progress

Methods for producing and screening illegitimate and transposon recombinants of *M. bovis* were developed (Wards and Collins, 1996; Wilson et al., 1997; Collins et al., 2002, 2005; Hotter et al., 2005), and a total of 81 mutants were selected for virulence testing in guinea pigs. A range of different methods was used for screening including signature tag mutagenesis, but most avirulent mutants were discovered from their inability to grow when inoculated from stationary phase cultures into minimal media. The results from this screening and the subsequent virulence testing are given in Table 1. A mutant was defined as avirulent if 10^6 cfu caused no visible tuberculous lesions in three guinea pigs eight weeks after sub-cutaneous inoculation (Collins et al., 2002).

The 25 mutants that were avirulent in guinea pigs were tested for their vaccine efficacy in groups of six guinea pigs per mutant using a low dose aerosol challenge (Collins et al., 2002). In comparison to BCG Pasteur, 14 of these avirulent mutants gave comparable protection and one

Table 1
Random mutants of *M. bovis* produced and tested for attenuation in guinea pigs.

Screening method	Mutants screened	Mutants selected	Partly attenuated	Avirulent
Signature tag	1200	37	11	4
Recovery from stationary phase	7500	27	24	16
Survival in alveolar macrophages	2200	7	7	3
Morphology	5000	4	2	1
Sensitive to cycloserine	2000	2	1	1
Sensitive to low oxygen	4000	4	2	0
Total	13,700[a]	81	47	25

[a] Total number of different mutants used; no mutants were screened by all methods.

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