

# **Immunology of possum biocontrol and bovine tuberculosis**

**Annual report for Biosecurity  
New Zealand 2006-07 (CO143)**

MAF Biosecurity New Zealand Technical Paper No: 2009/34

Prepared for MAFBNZ Operational Research  
By Bryce M. Buddle, AgResearch, Hopkirk Research Institute,  
Palmerston North

ISBN 978-0-478-35189-7 (Print)  
ISBN 978-0-478-35190-3 (Online)

ISSN 1176-838X (Print)  
ISSN 1177-6412 (Online)

**June 2007**



## Disclaimer

While every effort has been made to ensure the information in this publication is accurate, the Ministry of Agriculture and Forestry does not accept any responsibility or liability for error or fact omission, interpretation or opinion which may be present, nor for the consequences of any decisions based on this information.

Any view or opinions expressed do not necessarily represent the official view of the Ministry of Agriculture and Forestry.

The information in this report and any accompanying documentation is accurate to the best of the knowledge and belief of AgResearch Limited acting on behalf of the Ministry of Agriculture and Forestry. While AgResearch Limited has exercised all reasonable skill and care in preparation of information in this report, neither AgResearch Limited nor the Ministry of Agriculture and Forestry accept any liability in contract, tort or otherwise for any loss, damage, injury, or expense, whether direct, indirect or consequential, arising out of the provision of information in this report.

Requests for further copies should be directed to:

Strategic Science Team  
Policy and Risk Directorate  
MAF Biosecurity New Zealand  
P O Box 2526  
WELLINGTON

Telephone: 0800 00 83 33  
Facsimile: 04-894 0300

This publication is also available on the MAF website at  
[www.biosecurity.govt.nz/about-us/our-publications/technical-papers](http://www.biosecurity.govt.nz/about-us/our-publications/technical-papers)

© Crown Copyright - Ministry of Agriculture and Forestry

| <b>Contents</b>   | <b>Page</b> |
|---|-------------|
| <b>1 Objectives</b>   | <b>3</b>    |
| 1.1 Biocontrol of possums   | 3           |
| 1.2 TB vaccines for possums   | 3           |
| <b>2 Biocontrol of possums</b>  | <b>4</b>    |
| 2.1 Evaluate and optimise novel synthetic glycolipids in <i>in vitro</i> surrogate systems (dendritic cells, other antigen presenting cells) and mouse models for adjuvant activity for subsequent use in oral vaccines for possums | 4           |
| 2.2 Adjuvants and oral delivery systems for inducing antibody and cell-mediated immune responses in possums against key antigens associated with an anti-fertility immune response  | 6           |
| 2.3 Generation of bionanoparticles expressing a model antigen   | 8           |
| 2.4 Antigens from <i>Parastrongyloides trichosuri</i> for a diagnostic assay  | 12          |
| 2.5 Develop and validate tools to measure the immune responses in possums to antigens expressed by transgenic possum-specific nematodes.  | 14          |
| <b>3 TB vaccines for possums</b>  | <b>15</b>   |
| 3.1 Evaluate newly attenuated <i>Mycobacterium bovis</i> vaccine candidates for protection of possums against infection   | 15          |
| <b>4 Evaluation of the efficacy of oral bait BCG vaccine for wild possums</b>   | <b>19</b>   |
| <b>5 Publications in peer-reviewed scientific journals associated with this research contract (July 2006 – June 2007)</b>   | <b>20</b>   |



# Annual report for Biosecurity New Zealand - 2006-07

**Biosecurity NZ contract:** 0307C01  
**Objective title:** Immunology of possum biocontrol and bovine tuberculosis  
**Research Leader:** Dr Bryce Buddle, AgResearch

## SUMMARY

### Biocontrol of possums

For delivery of a vaccine to wild possums, administration by the oral route as a bait would be the most practical means. However, it is very difficult to induce a strong immune response with a non-living vaccine delivered orally and an effective adjuvant must be incorporated into the vaccine for any chance of success. Studies were carried out to evaluate some synthetic glycolipids in *in vitro* assays and in mice as possible adjuvants, which could be used for future testing in possums. A number of these synthetic glycolipids induced IL-12 cytokine responses in mouse and cattle dendritic cells, which indicated that they had potential to stimulate cellular immunity. This was confirmed in mouse studies where these glycolipids stimulated cellular responses when administered by oral or systemic routes of administration, but stimulated little or no antibody. These results suggest that the glycolipids would best be used for tuberculosis vaccines, which require strong cellular immunity rather than for an immunosterilising vaccine. A study was carried out in possums to vaccinate against gonadotropin releasing hormone (GnRH) which potentially could interfere with the reproductive ability of possums. GnRH is a very small molecule and must be coupled to a larger carrier protein (keyhole limpet haemocyanin) or a particle such as bionanoparticle to stimulate any immune responses. Vaccines were administered by injection or orally to possums. When GnRH was conjugated to the carrier protein and delivered by injection, strong systemic cellular and antibody responses were generated to the carrier protein as well as an antibody response to the carrier protein in the uterine and vaginal secretions, but no immune responses to GnRH. Similarly, when GnRH was conjugated to bionanoparticles, an immune response was generated to the bionanoparticle, but not to GnRH. These results suggest that the binding efficiency of GnRH to the carrier protein or bionanoparticle would need to be enhanced, but it was encouraging that mucosal antibody responses could be induced when vaccines are delivered systemically. Although the vaccine delivered orally included two known mucosal adjuvants, no immune responses were observed in animals vaccinated with this vaccine. Progress has been made in the expression of model antigens in bionanoparticles and this is an encouraging vaccine delivery system for the future.

The possum-specific nematode, *Parastrongyloides trichosuri* is a possible vector for delivery of an immunosterilising vaccine. Studies were undertaken to identify candidate antigens from this nematode that could be used in an ELISA assay to measure immune responses to this parasite and several potential antigen candidates were identified. In a second study, *P. trichosuri* was engineered to contain a foreign gene as preliminary step in the generation of a transgenic nematode that could be used for biocontrol of possums. Possums were infected with these nematodes and although the relative rate of transgenesis in the nematodes was low, several of the possums induced a lymphocyte proliferation response to the protein expressed by the foreign gene.

### TB vaccine for possums

A number of newly derived attenuated *Mycobacterium bovis* strains were evaluated for their ability to protect possum from an experimental challenge with virulent *M. bovis*. Two of these

strains produced very encouraging results with lung pathology and bacterial counts lower than those observed in possums vaccinated with the standard BCG vaccine. Progress is being made to gain registration of an oral bait BCG vaccine for possums and a field trial was conducted in the Orongorongo Valley, near Wellington to determine whether wild possums receiving orally administered BCG could be protected against natural exposure to tuberculosis. The study extended over a 2-year period and involved 104 vaccinated and 130 control possums. The possum population was monitored on 2-monthly basis by trapping in cage traps, examining for tuberculous lesions and then release. At the end of the study period, those possums that could be caught were killed and were carefully examined. A total of 13 possums were found to have tuberculosis, confirmed by culture of *M. bovis*. Vaccination with BCG had a significant protective effect with 12 of the infected possums being in the control group and only one in the BCG-vaccinated group. This finding indicates that vaccination of possums could be a very useful tool in the control of tuberculosis in wild possums.

# 1 Objectives

## 1.1 BIOCONTROL OF POSSUMS

- Evaluate and optimise novel synthetic glycolipids in *in vitro* surrogate systems (dendritic cells, other antigen presenting cells) and mouse models for adjuvant activity for subsequent use in oral vaccines for possums.
- Determine the efficacy of selected oral adjuvants, alone or in combination with permeability increasing agents for the induction of antibody and cell-mediated immune responses in possums against key antigens associated with an anti-fertility immune response.
- Produce and test bionanoparticles containing a model antigen. These bionanoparticles will be assessed for their potential to stimulate immune responses to specific antigens. If successful these particles could be used to deliver antigens relevant for biocontrol of possums.
- Purify and identify immunogenic antigens from a possum-specific nematode, *Parastrongyloides trichosuri* for the development of an ELISA serological test.
- Develop and validate tools to measure the immune responses in possums to antigens expressed by transgenic possum-specific nematodes. The transgenic nematodes would serve as a vector for an immunosterilising vaccine.

## 1.2 TB VACCINES FOR POSSUMS

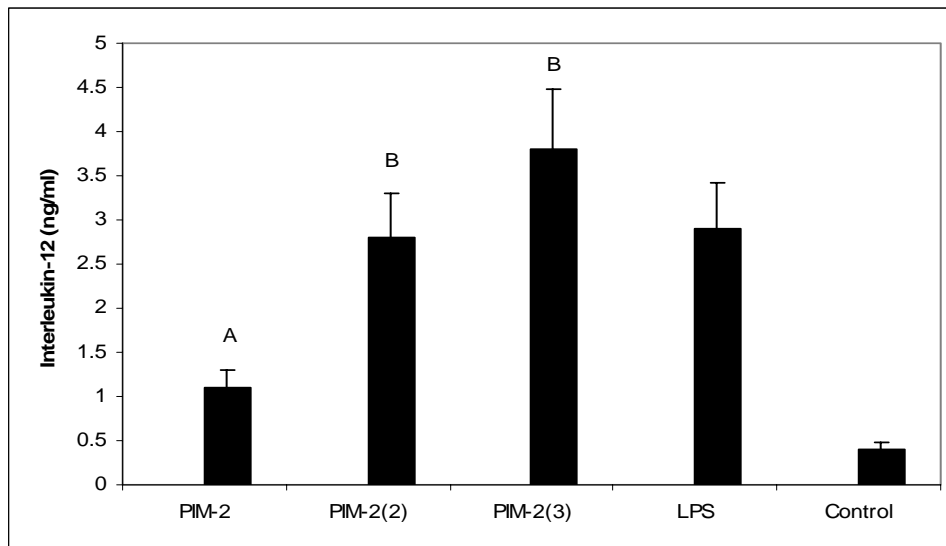
- Evaluate newly developed attenuated *Mycobacterium bovis* vaccine candidates developed by Dr Des Collins, AgResearch for protection of possums against *M. bovis* infection.
- Complete studies on the efficacy of oral bait BCG vaccine to protect wild possums against tuberculosis in the Orongorongo Valley against natural challenge with *M. bovis* infection. This is a collaborative project in association with Landcare Research, which commenced in November 2004, and the trial is scheduled to end in late 2006.

## 2 Biocontrol of possums

### 2.1 EVALUATE AND OPTIMISE NOVEL SYNTHETIC GLYCOLIPIDS IN *IN VITRO* SURROGATE SYSTEMS (DENDRITIC CELLS, OTHER ANTIGEN PRESENTING CELLS) AND MOUSE MODELS FOR ADJUVANT ACTIVITY FOR SUBSEQUENT USE IN ORAL VACCINES FOR POSSUMS

We have tested an array of novel pure synthetic glycolipids, related to products from the mycobacterial cell walls, which have inherent adjuvant properties. Some of the products are unique from a chemical perspective. Phosphatidylinositol mannoside (PIMs) are glycolipids embedded in the cell wall of mycobacteria, and anchor more complex glycolipids to the cell membrane. We are interested in these molecules from two perspectives: the first one is the ability of these molecules to induce a cell-mediated response, an important part of our work on developing and discovering novel ways to optimize vaccination procedures of possums against bovine tuberculosis. Secondly, these molecules may assist in stimulating a substantial antibody response, which is related to our continuing work on defining and optimizing formulations for an anti-fertility vaccine in possums. Adjuvants that have strong immune promoting activities generally do so directly or indirectly by inducing the release of interleukin-12 (IL-12) by antigen-presenting cells, most notably dendritic cells (DCs) which present antigens, and drive the proliferation of T cells that respond to the antigens administered. From the perspective of our work, mouse DCs and bovine DCs are readily isolated using recombinant cytokines from the mouse and bovine species. However, such recombinant cytokines are not available for possums, although they will become available as the newly derived EST library for the brushtail possum is being constructed, and the whole array of possum cytokine genes are identified, (<http://www.possumbase.org.nz/cgi-bin/gbrowse/mondom4/>).

Preliminary work was undertaken in cattle, due to the ease of obtaining blood samples and DCs. Figure 1 shows the potent IL-12 inducing activities of the novel PIM analogues we have been studying. These data with PIMs are in agreement with recent data on a vaccinating trial where we are studying different adjuvants to enhance the immunogenicity of vaccines for protection against bovine tuberculosis. Indeed, preliminary results indicate that PIMs result in the production of substantial amounts of interferon-gamma (IFN- $\gamma$ ) following vaccination, a good indicator that a substantial immune response is developing in PIM-treated animals, (DEFRA contract, Buddle et al, unpublished observations). LPS was used as a control, as it is one of the most potent IL-12 inducer, but it cannot be used as an adjuvant because of its high toxicity.



**Figure 1. IL-12 inducing ability of synthetic PIM-2 and synthetic PIM-2 analogues.** Columns identified by different letters are significantly different from each other. PIM-2 refers to regular synthetic acylated PIMs, whereas PIM-2(2) and PIM-2(3) refer to PIMs with ether linkages at the 2 and 3 position, respectively. LPS (lipopolysaccharide) is used as positive control for induction of IL-12.

We have made substantial progress in showing that phosphatidylinositol dimannoside (PIM2, 1) ether analogues (2) and (3) are excellent IL-12 inducers in bovine DCs.

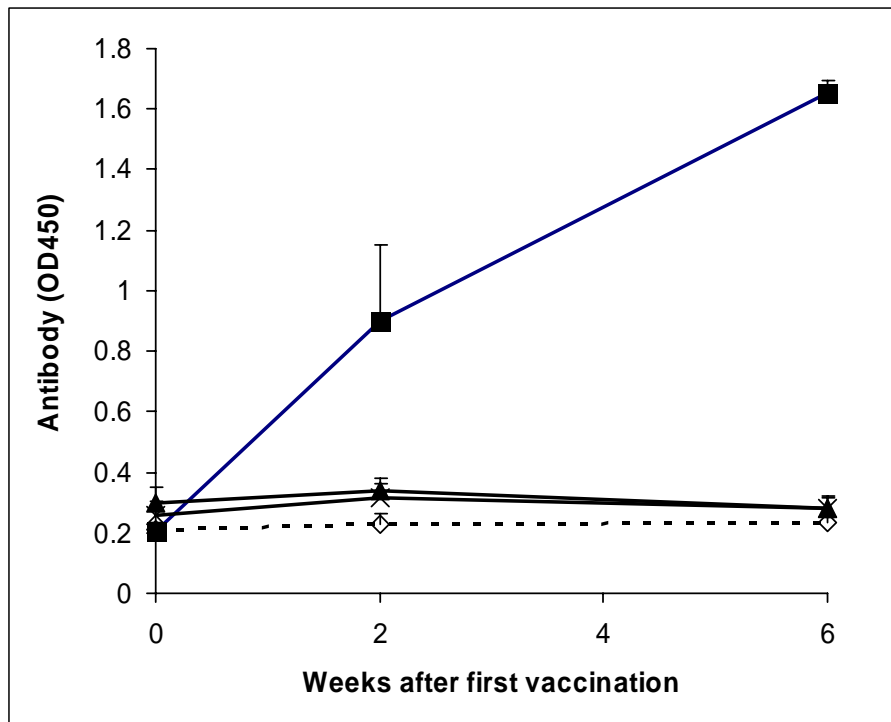
Our recent experiments using OT-II mice have shown the synthetic glycolipid PIM has an adjuvant effect promoting a Th1 type response, specifically an increase in IFN- $\gamma$  and no Th2 cytokines. The transgenic model used only one vaccination, to allow for rapid screening of the molecules. Our current approach is to use non-transgenic mice (regular B6 mice) and giving 3 vaccinations with a model antigen, ovalbumin (OVA), 1 week apart. We have modified the original protocol to allow us to eventually apply these findings to our possum trials on oral vaccination, notably to induce a substantial antibody response to anti-fertility vaccines in possums. PIM2 or variants of PIM2 were used as adjuvants. Cholera toxin was used as a positive control adjuvant. Two weeks after the 3<sup>rd</sup> immunisation, mesenteric lymph nodes and spleens were cultured with OVA peptide as restimulating antigen and culture supernatants analysed for cytokine production. Blood was taken and antibody isotype responses were measured by ELISA. Using non-transgenic mice there was no evidence of an IgG antibody response. Use of PIM2 in vaccines showed an increase in the lymphocyte proliferative response to ovalbumin at 2 weeks post vaccination. These results suggest that the glycolipids would best be used for tuberculosis vaccines, which require strong cellular immunity rather than for an immunosterilising vaccine.

## 2.2 ADJUVANTS AND ORAL DELIVERY SYSTEMS FOR INDUCING ANTIBODY AND CELL-MEDIATED IMMUNE RESPONSES IN POSSUMS AGAINST KEY ANTIGENS ASSOCIATED WITH AN ANTI-FERTILITY IMMUNE RESPONSE

Vaccines that interfere with gonadotropin releasing hormone (GnRH) activity are candidates for interfering with the reproductive ability of possums. An efficient oral delivery system will need to be developed in order for a GnRH based vaccine to have practical application for biocontrol of possums. However, it is difficult to induce strong immune responses against antigens administered by the oral route and this is necessary as vaccines delivered to wild possums are most practically delivered as oral baits. Our aim has been to identify adjuvants and devise a suitable delivery system for a bait-delivered fertility control vaccine in possums. A lipid-based formulation has been developed as a vehicle for oral delivery of the BCG vaccine. Live BCG is incorporated into a lipid matrix and upon subsequent oral delivery, the lipid matrix is thought to aid bacterial survival in the stomach and proximal small intestine, allowing live BCG bacteria to reach sites of immune induction in the small intestine (the Peyer's patches). The lipid may act to protect a protein based vaccine from acid degradation. Two adjuvants,  $\beta$ -glucan and chitosan have been shown to have an effect at mucosal surfaces and therefore were considered candidate adjuvants for generating mucosal immunity in possums.

An experiment was conducted to determine whether immunisation with an orally administered GnRH vaccine formulated with the protective lipid and incorporating  $\beta$ -glucan and chitosan could stimulate systemic and mucosal immune responses (both antibody and cell-mediated in the possum). We prepared vaccines by cross linking GnRH peptide onto a carrier protein, keyhole limpet haemocyanin (KLH) using 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). Five possums (4 female and 1 male) were immunised by the oral route with 150  $\mu$ g GnRH cross-linked to KLH + lipid and adjuvants ( $\beta$ -glucan and chitosan). A second group (3 females and 1 male) were immunised subcutaneously with GnRH cross-linked to KLH (150  $\mu$ g) + Freund's Complete Adjuvant FCA (n=4), and a control group (4 females) were non-immunised. In addition, a fourth group of possums (4 female and 1 male) were immunised subcutaneously with 250  $\mu$ g of a vaccine prepared by cross-linking GnRH to bionanoparticles. This vaccine was co-administered with the oil-in-water adjuvant, Emulsigen<sup>TM</sup>. A full account of the production and testing of bionanoparticles is described in more detail in the next section. The possums were re-immunised with the same vaccines at 3 weeks and sacrificed at 6 weeks. Blood and sera were collected prior to immunisation and post-immunisation. At sacrifice, reproductive samples (from uteri and vagina were obtained from the female possums to measure antibody levels at mucosal sites. The proliferation response of peripheral blood lymphocytes to antigens (KLH and GnRH) and the level of antibodies in sera and at female reproductive sites were determined.

Vaccination of possums subcutaneously with the cross-linked GnRH/KLH vaccine induced strong serum antibody responses to KLH (Figure 2). In addition, significant levels of antibody to KLH were also detected in the secretions from the uteri and vagina of the female possums administered with this vaccine.



**Figure 2. Mean ( $\pm$  SEM) antibody responses to KLH in sera of possums vaccinated with GnRH vaccines: oral GnRH/KLH (\*), subcutaneous GnRH/KLH (■) subcutaneous GnRH/bionanoparticles (▲), non-vaccinated (◇).**

Subcutaneous vaccination of possums with GnRH/KLH induced strong cellular responses to KLH (data not shown). Administration of GnRH/KLH by the oral route induced only minimal responses. None of the vaccine tested induced significant cellular or antibody responses to GnRH.

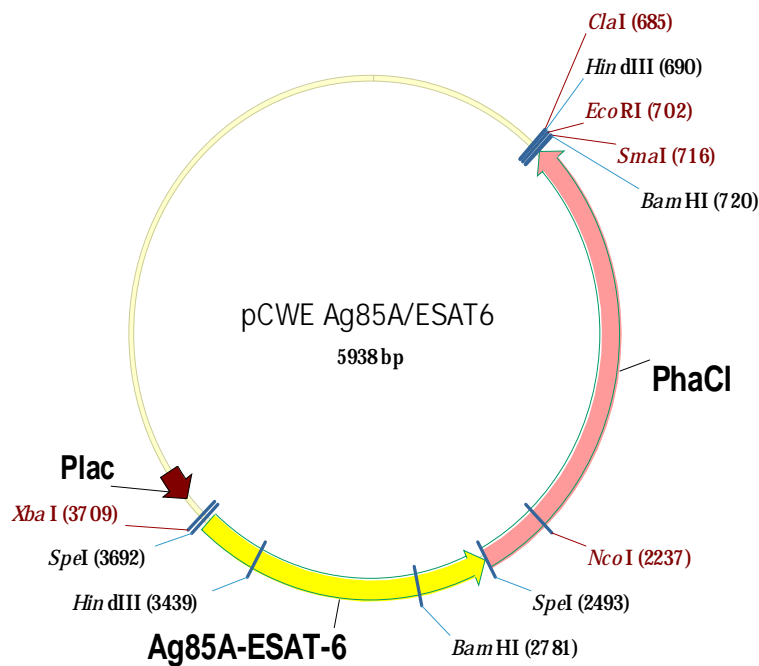
These results highlight the difficulty of inducing an immune response to an antigen when delivered by the oral route. It is encouraging that generation of a systemic antibody response in possums, as was induced by subcutaneous administration of GnRH/KLH can also produce antibody at a mucosal site relevant to biocontrol.

## 2.3 GENERATION OF BIONANOPARTICLES EXPRESSING A MODEL ANTIGEN

Biocontrol agents and vaccines are currently being developed for control of brushtail possums and are likely to be delivered by a mucosal route as oral bait or by nasal sprays. Delivery of biocontrol agents and vaccines could be more effectively achieved using nanoparticle technology. Recent technology developed at Massey University has shown that biopolyesters (bionanoparticles) are produced by a number of different bacteria and deposited as water-insoluble inclusions in the cytoplasm. Methods have been developed to manipulate microbes to control the formation of these bionanoparticles and this has led to their development as carriers of active molecules. Antigens, immunomodulators and adjuvants can be incorporated into the bionanoparticles, either in the particle lumen or on the surface making these particles attractive as delivery vehicles for vaccines and drugs. A particular advantage of this system is co-delivery of antigen and immune modulator to the same antigen presenting cell.

We plan to develop bionanoparticles as a vehicle for delivering antigens to possums for the purpose of biological control and for delivering *Mycobacterium bovis* antigens as a more effective vaccine against tuberculosis. A model antigen, a fusion of two immunodominant mycobacterial antigens Ag85A and ESAT-6 was expressed in bionanoparticles. The ability of engineered bionanoparticles expressing this fusion protein will be tested in *in vitro* assays and *in vivo* using mouse models. Knowledge from the mouse models will be used to produce bionanoparticles for delivery of relevant biocontrol antigens to possums such as GnRH or the Zona pellucida ZP3 protein. An ultimate aim of the project will be the development of optimised bionanoparticle-based delivery systems for biocontrol of possums against tuberculosis.

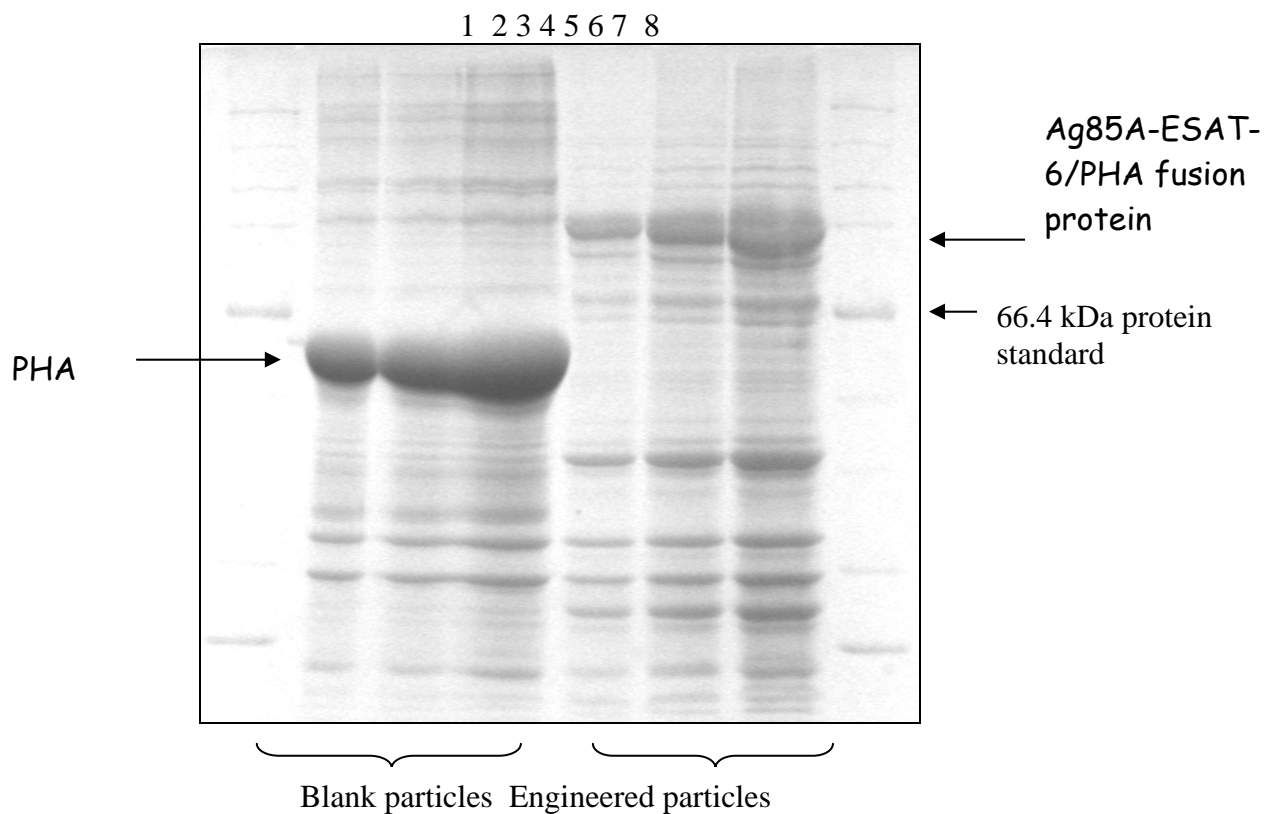
A system for genetic manipulation of bacteria for production of engineered bionanoparticles carrying protein molecules of interest has been devised by Prof Bernd Rehm at Massey University. Plasmids carrying essential components of polyhydroxyalkanoate (PHA) (biopolyester particles) synthesis such as PHA synthase genes have been developed. We cloned the Ag85A-ESAT-6 fusion gene into the plasmid, pCWE at the *SpeI* restriction site to create a fusion between the mycobacterial gene and the PHA synthase gene PhaC1 (Figure 3).



**Figure 3. Schematic diagram of construct to express Ag85A-ESAT-6 in bionanoparticles**

This construct was transformed into a production strain of *E. coli* to produce bionanoparticles. The yield of bionanoparticles produced from bacteria harbouring this plasmid was poor. Efficient expression of the mycobacterial protein/PHA fusion may require transcription of the gene from a stronger promoter than Plac. A further construct, based on the pET-14b plasmid (Novagen) which has the strong T7 promoter was made. An XbaI-ClaI fragment, containing the Ag85A-ESAT-5/PhaCI fusion gene was excised from the pCWE Ag85A/ESAT6 construct and sub-cloned into pET-14b at the XbaI and ClaI sites. *E. coli* transformed with this construct produced large numbers of bionanoparticles. The presence of bionanoparticles within the bacterial cells was verified by microscopical visualisation and the expression of the PHA-mycobacterial fusion protein on the bionanoparticles was confirmed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

Figure 4 shows the presence of PHA and PHA fusion protein at high levels within cell transformed with plasmid alone (blank bionanoparticles) and the construct expressing the Ag85A-ESAT-6/PHA fusion protein.

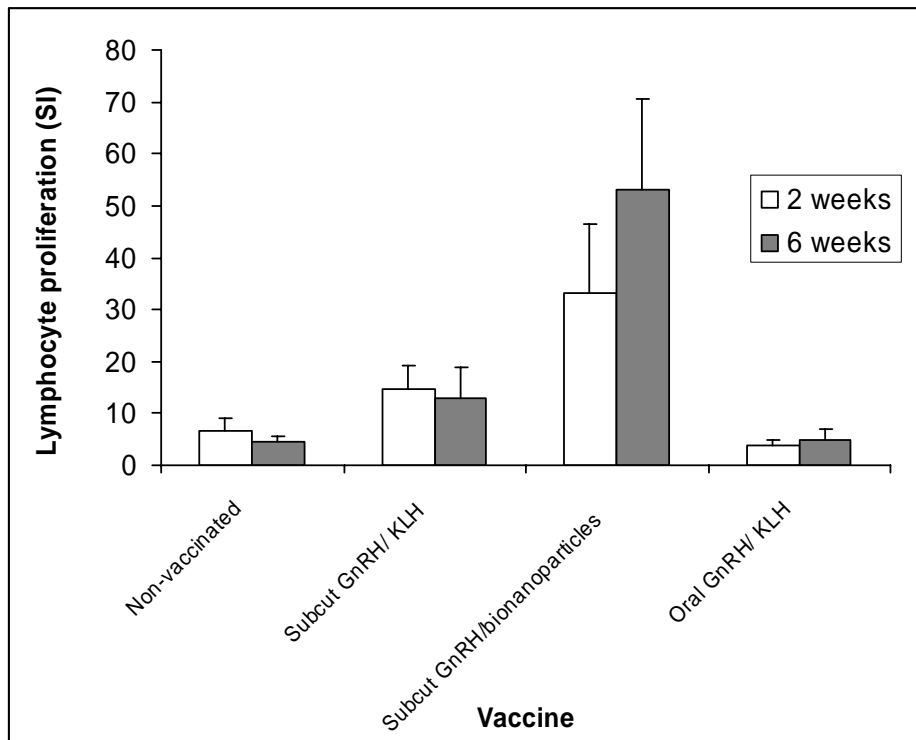


**Figure 4. Expression of PHA in blank bionanoparticles and Ag85A-ESAT-6/PHA fusion protein in the engineered bionanoparticles**

The particles expressing the PHA/mycobacterial fusion protein will be tested further in an *in vitro* assay using blood from tuberculosis infected cattle and in a mouse model of tuberculosis infection to determine ability to generate immune responses against the mycobacterial antigens.

In addition to the genetic approach to engineering bionanoparticles, the desired antigens could be cross-linked onto the surface of the bionanoparticle. We explored this possibility to produce a bionanoparticles delivery system for the biocontrol agent Gonadotropin releasing hormone (GnRH). GnRH was cross-linked onto blank bionanoparticles using 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC). Possums (4 female and 1 male) were immunised subcutaneously with 250 µg of the GnRH cross-linked bionanoparticles administered along with the oil-in-water adjuvant, Emulsigen™ as described above. Non-vaccinated possums (n=5) served as controls. Cellular responses to GnRH and KLH were measured and, additionally cellular responses to blank bionanoparticles were determined in the *in vitro* lymphocyte proliferation assay.

As was the case with the GnRH cross-linked onto KLH, the GnRH/bionanoparticles induced minimal cellular and antibody responses to GnRH. The cross-linking of the GnRH to the bionanoparticles may not be optimal and a better approach to presenting GnRH peptide on the surface of the bionanoparticles may be to produce GnRH bionanoparticles by the genetic approach, as was performed for the Ag85A-ESAT-6 protein. In the *in vitro* lymphocyte proliferation assay, in addition to using GnRH and KLH as re-stimulating antigen, blank bionanoparticles were used. The possums vaccinated with the bionanoparticle vaccine produced significant proliferative responses to bionanoparticles in the *in vitro* assay (Figure 5). This suggests that the bionanoparticles have intrinsic immunomodulatory properties; the precise nature of this function is presently unknown.



**Figure 5. Mean ( $\pm$  SEM) proliferative responses to blank bionanoparticles in possums vaccinated with GnRH vaccines: subcutaneous KLH/bionanoparticles, subcutaneous GnRH/KLH, oral GnRH/KLH, or non-vaccinated.**

## 2.4 ANTIGENS FROM *PARASTRONGYLOIDES TRICHOSURI* FOR A DIAGNOSTIC ASSAY

The possum-specific nematode, *Parastrongyloides trichosuri* is being considered as a possible vector for a transmissible immunosterilising vaccine. Foreign genes can be inserted and expressed successfully in this nematode. In order to develop *P. trichosuri* as a transmissible biocontrol agent and to monitor the release and effectiveness of a *P. trichosuri* based vaccine it will be necessary to measure serological responses to this organism using an ELISA assay. Previous use of an ELISA with crude extracts of this nematode has not proven to be specific and there is a requirement for an ELISA based on a purified antigen or antigens.

Our aim has been to develop an antigen-specific ELISA serological test for *P. trichosuri*. A number of extracts were prepared from *P. trichosuri* L3 larvae using detergents to release surface antigens, molecules likely to be immunogenic. Surface antigen extracts were prepared using the anionic detergent, sodium deoxycholate and a cationic detergent, hexadecyltrimethylammonium bromide. Extracts were also prepared using urea and phenol. Each of these extracts was tested for their potential as diagnostic agents in an ELISA using a panel of sera from possum infected with *P. trichosuri* (n=10). As controls, sera were obtained from *P. trichosuri* negative possums, including sera from 10 possums sourced from an area of Southland where *P. trichosuri* infection is not prevalent.

An ELISA was developed to measure anti-Pt antibodies in the possum sera against the various Pt fractions. Figure 6 shows the mean antibody levels (expressed as absorbance at 450 nm) in the positive and negative sera against the fractions. Each of these fractions containing *P. trichosuri* surface antigen has potential to distinguish between Pt infected and non-infected possums.

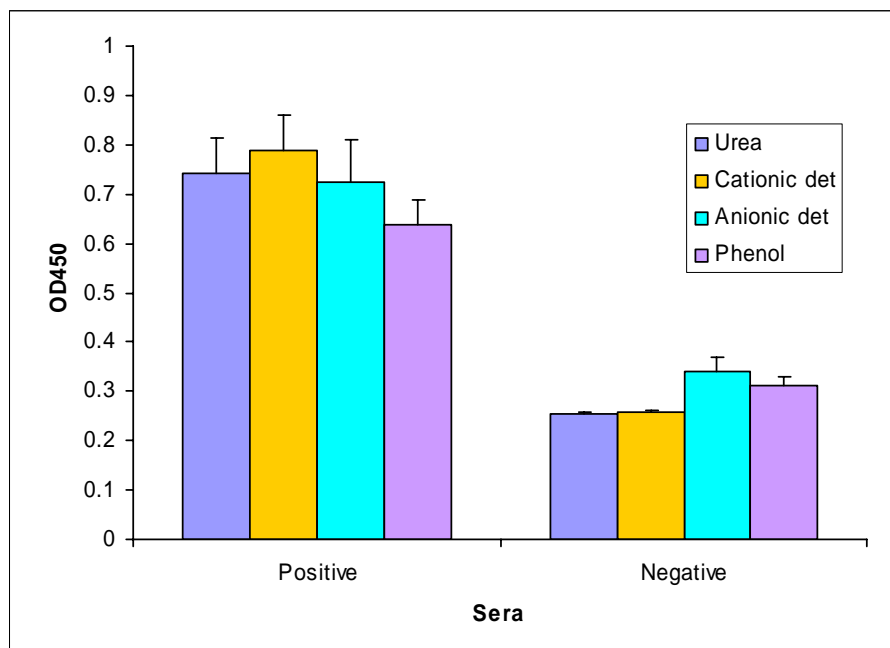
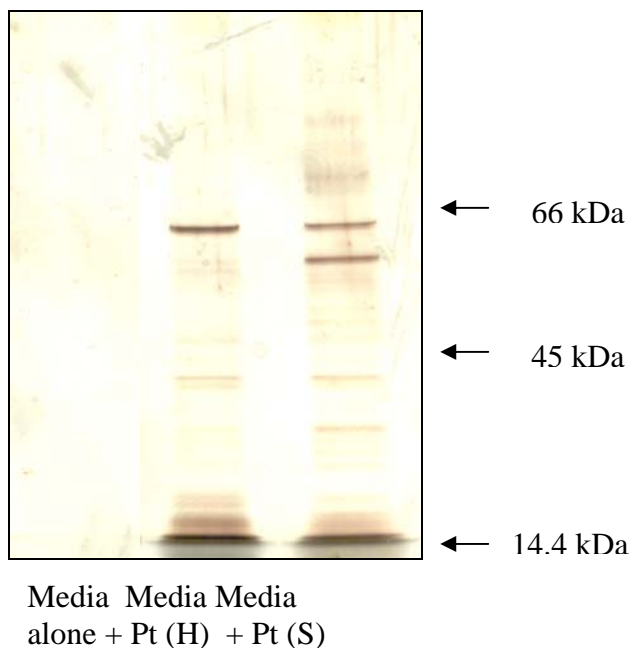


Figure 6. Mean antibody responses against *P. trichosuri* fractions

Another approach has been to identify antigens released or secreted during growth of the *P. trichosuri* as these may be recognised more effectively by the immune system compared to surface antigens or antigens which are an integral part of the organism. *P. trichosuri* were isolated from previously infected possums using two different methods, hanging possum intestines to flush out the nematodes and physical scraping of intestines. Intestinal material from the guts of a possum previously infected with *P. trichosuri* and then treated with anthelmintics was also cultured in media to serve as a control. The nematodes were cultured in DMEM at 37°C for a period when most of the organisms retained viability. After 24 hours incubation, the media was recovered by centrifugation, concentrated by ultrafiltration (molecular weight cut-off 10 kDa) and analysed by SDS-PAGE.

Figure 7 shows the presence of proteins in media conditioned with *P. trichosuri*. No proteins were detected in DMEM medium alone suggesting that some or all of these proteins have been released from *P. trichosuri* during culture and therefore are potential candidates for developing a diagnostic system for determining *P. trichosuri* infection in possums.



H: nematodes obtained by gut hanging; S: nematodes obtained by gut scraping.

**Figure 7. SDS-PAGE analysis of proteins present in culture media conditioned with *P. trichosuri* (silver stained gel)**

In order to determine whether these proteins released into the media could help differentiate between infected and non-infected possums, Western blotting was performed. The proteins separated by SDS-PAGE were immunoblotted to PVDF membranes and exposed to *P. trichosuri* positive possum sera (three of the sera used in the ELISA were pooled for this purpose). A protein A:horse radish peroxidase conjugate was used as the secondary antibody. The pooled sera only reacted weakly and no *P. trichosuri* specific bands were identified. This work can be repeated using a range of high titre sera from *P. trichosuri*-infected possums.

## 2.5 DEVELOP AND VALIDATE TOOLS TO MEASURE THE IMMUNE RESPONSES IN POSSUMS TO ANTIGENS EXPRESSED BY TRANSGENIC POSSUM-SPECIFIC NEMATODES.

Current research in the Possum Biocontrol OBI involves the insertion of foreign genes into the possum-specific nematode, *P. trichosuri* to test whether this nematode could serve as a vector for an immunosterilising vaccine. The production of a transgenic *P. trichosuri* has been achieved by the AgResearch Parasitology group lead by Dr Warwick Grant and the next step is to determine the infectious load of the transgenic nematode necessary to generate an immune response in a possum to the protein expressed by the foreign gene. An experiment was undertaken to infect possums with two different lines of transgenic nematodes and to measure both antibody and cellular immune responses to the foreign protein. The foreign was a fusion of two mycobacterial genes, Ag85a and ESAT-6. A group of six possums were each infected intradermally with 5,000 larvae from line C1 transgenic *P. trichosuri* and a further 2,000 larvae on day 4. A second group of possums were infected with line C2 transgenic *P. trichosuri* in a similar manner as for the first group. A third group of six possums were infected with non-transgenic *P. trichosuri* (control group). Blood samples were collected from all possums at day 0, 21 and 42 and faecal egg counts were monitored on a regular basis. All possums were killed on day 42. Mesenteric lymph nodes were collected to study mucosal lymphocyte proliferation (LP) responses to Ag85a/ESAT-6 and intestinal contents were collected to determine the number of transgenic nematodes present as well as mucosal antibody responses.

There was a low rate of transgenesis (7-10%) in the nematode population recovered from the possums infected with the transgenic nematodes. This may be partially due to loss of the plasmid containing the Ag85a/ESAT-6 with multiple generations of the transgenic nematodes. There were no antibody responses to Ag85a/ESAT-6 detected in the serum, intestinal content or faecal pellet. Although, there were no significant differences between the peripheral blood LP responses to Ag85a/ESAT-6 for the three groups of possums, there were a number of possums in the groups receiving the transgenic nematodes that produced positive LP responses to Ag85a/ESAT-6. The positive cut-off (4.4 stimulation index, SI) was set as the mean LP value prior to infection for all possums + three standard deviations. No possums had positive responses prior to infection or in the control group at any time, while two possums from line C1 group and one possum from line C2 group were positive at 21 days and two possums from line C1 were positive at day 42. No positive LP responses to Ag85a/ESAT-6 were observed from lymphocytes cultures prepared from the mesenteric lymph nodes.

Considering the low level of transgenesis in the nematode population recovered from the possums, the LP results were encouraging with several animals producing immune responses to the foreign protein. The technique of recovering lymphocytes from the mesenteric lymph nodes was successful and the majority of the lymphocyte cultures responded strongly to the mitogen. Future work for the Parasitology group is focussed on finding a method that results in a more stable integration of the foreign gene into the *P. trichosuri* nematode.

## 3 TB vaccines for possums

### 3.1 EVALUATE NEWLY ATTENUATED *MYCOBACTERIUM BOVIS* VACCINE CANDIDATES FOR PROTECTION OF POSSUMS AGAINST INFECTION

The current bovine tuberculosis control programme has dramatically reduced the number of cattle herds infected with *Mycobacterium bovis*. However, this strategy has had little effect in reducing the proportion of areas in New Zealand (40 percent) where tuberculosis-infected possums are found. Additional disease control tools are needed and an effective vaccine to eliminate *M. bovis* infection in possums remains an attractive option. The human tuberculosis vaccine, BCG does not induce complete protection against tuberculosis in possums and there is an urgent need to develop better vaccines. A number of newly attenuated *M. bovis* vaccine candidates (WAg strains) have been developed by our collaborator Des Collins at AgResearch Wallaceville (FRST-funded project). These candidates have been shown to be attenuated following inoculation in guinea pigs and possums. In addition, a number of these strains have been shown to be equivalent or slightly better than BCG in vaccine efficacy trials in guinea pigs.

Two vaccination studies were conducted in possums using these new attenuated *M. bovis* strains. In the first study, the groups of possums were vaccinated subcutaneously with three of these strains and these possums were challenged with an aerosol of virulent *M. bovis* to determine protection against tuberculosis. In the second study, three new attenuated strains were fed to groups of possums to assess whether strong immune responses could be induced when the vaccines are delivered orally.

#### 3.1.1 Study 1

Three of these new attenuated strains were used as vaccines and the efficacies of these vaccines were tested in possums following subcutaneous injection and compared to BCG vaccine.

- Four groups of possums (6 animals/group) were vaccinated subcutaneously with approximately  $5 \times 10^5$  CFU of live bacteria comprising either *M. bovis* WAg559, WAg563, WAg584 or Pasteur BCG strain.
- A fifth groups of possums (n=7) served as non-vaccinated controls.
- At 8 weeks after vaccination, the possums were challenged with an aerosol of virulent *M. bovis* using an aerosol-generating chamber.
- Seven weeks after challenge all possums were killed and necropsied.

Vaccination with all of the new attenuated *M. bovis* vaccines and BCG induced strong lymphocyte proliferation (LP) responses to bovine PPD at 5 and 8 weeks after vaccination. The positive LP cut-off was set from the mean for all groups prior to vaccination (stimulation index of 2.0) plus 3 standard deviations (SD) ( $3 \times 1.8$ ) and the cut-off was 7.4. All vaccinated possums had a stimulation index  $> 7.4$  at both 5 and 8 weeks after vaccination, whereas all of the non-vaccinated possums had LP responses of  $< 7.4$  at these time-points. The mean stimulation indices for the WAg559, WAg563, WAg584 or Pasteur BCG groups at 5 weeks post-vaccination were 104, 112, 89 and 65 and at 8 weeks, 123, 118, 61 and 91, respectively. All of the vaccinated groups had statistically stronger responses than the non-vaccinated group at both 5 and 8 weeks post-vaccination. At 5 weeks post-vaccination, responses were stronger for the WAg559 group compared to the Pasteur BCG group and at 8 weeks post-

vaccination, responses were stronger for the WAg563 group compared to the WAg584 group ( $P < 0.05$ , Mann Whitney U-test, pair-wise comparisons).

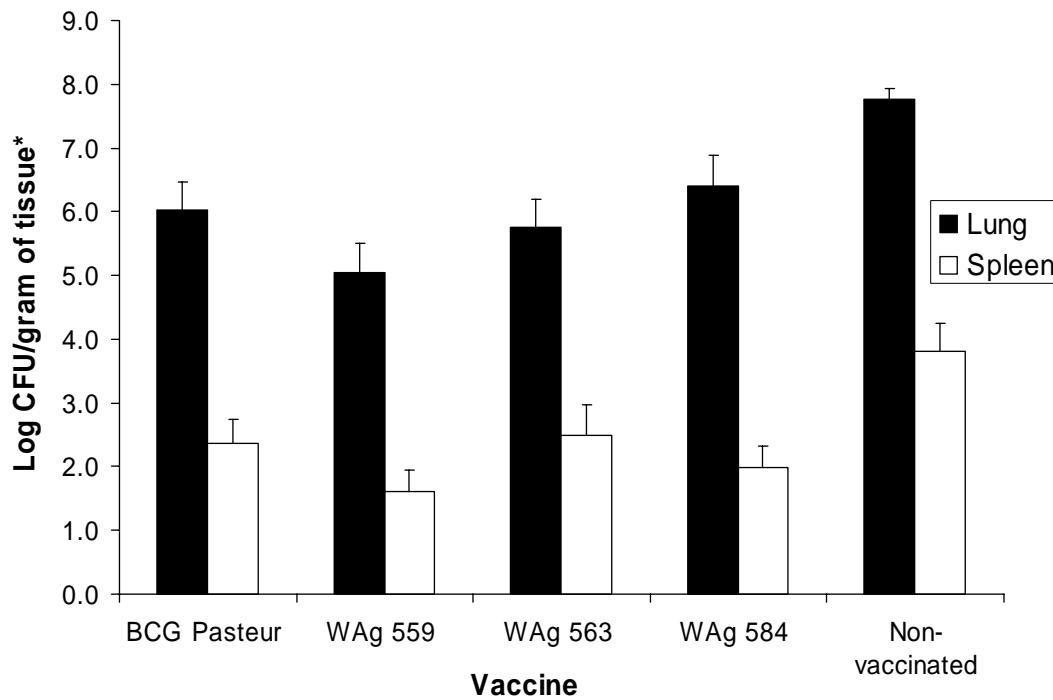
In the assessment of protection against tuberculosis, all vaccinated groups had significantly lower mean body weight losses between challenge and necropsy as a percentage of their body weight at challenge compared to the non-vaccinated group ( $P < 0.05$ , ANOVA, Table 1). The extent of lung pathology can be assessed from the lung weight as a percentage of body weight at challenge (heavier lung weight is associated with greater lung pathology). All vaccinated groups had significantly lower mean lung weights as a percentage of body weight compared to the non-vaccinated group ( $P < 0.05$ , ANOVA, Table 1). The bacterial counts followed a similar trend with significantly lower mean lung and spleen bacterial counts for all of the vaccinated groups compared to the means for the non-vaccinated group. In addition, the WAg559 group had a significantly lower mean lung bacterial count compared to the WAg584 group ( $P < 0.05$ , ANOVA on  $\log_{10}$  transformed data, Figure 8).

In summary, all of the vaccinated groups induced a significant level of protection in possums against challenge with *M. bovis*. Two of the newly attenuated *M. bovis* vaccine strains, WAg559 and WAg563 looked very promising.

**Table 1. Loss in body weight and extent of lung lesions in possums following aerosol challenge with *M. bovis***

| Vaccine        | % weight loss/body weight at challenge<br>( $\pm$ SEM) <sup><math>\alpha</math></sup> | % lung wt/body weight at challenge ( $\pm$ SEM) |
|----------------|---|---|
| BCG Pasteur    | 1.88 $\pm$ 4.86   | 1.358 $\pm$ 0.251                               |
| WAg559         | -3.68 $\pm$ 4.23  | 1.059 $\pm$ 0.128                               |
| WAg563         | -4.67 $\pm$ 2.09  | 0.768 $\pm$ 0.082                               |
| WAg584         | 4.67 $\pm$ 2.99   | 1.415 $\pm$ 0.056                               |
| Non-vaccinated | 18.74 $\pm$ 2.82  | 2.704 $\pm$ 0.394                               |

<sup>$\alpha$</sup>  % body weight loss: Body weight at challenge – body weight at necropsy divided by body weight at challenge and expressed as a percentage



\* bacterial count, CFU log<sub>10</sub>/g of tissue (± SEM)

**Figure 8. Lung and spleen bacterial counts following challenge of possums with *M. bovis***

### 3.1.2 Study 2

Three attenuated *M. bovis* strains (WAg539, WAg563 and WAg771) were assessed for their ability to induce strong cellular immune responses when delivered orally.

- Four groups of possums (6 animals/group) were fed two pellets containing approximately  $2 \times 10^8$  CFU of live bacteria comprising either *M. bovis* WAg539, WAg563, WAg771 or Pasteur BCG strain. The bacteria were incorporated into a lipid matrix to protect the bacteria from degradation in the stomach of the possum.
- A fifth groups of possums (n=7) served as non-vaccinated controls.
- At 0, 6 and 8 weeks after vaccination, blood samples were collected from all the possums and cellular immune responses to *M. bovis* antigens (bovine PPD) were measured using a lymphocyte proliferation assay.

All of the vaccines induced strong lymphocyte proliferation (LP) responses to bovine PPD (Table 2). None of the possums had LP responses above the positive cut-off prior to vaccination, while after vaccination between 5 to 6 possums from each of the vaccinated groups produced immune responses above the cut-off at either 6 or 8 weeks after vaccination. Feeding the possums with the vaccines incorporated into an oral bait was shown to be an effective means of immunising the possums and a vaccination/challenge trial is now planned to test the effectiveness of these three new attenuated vaccine delivered as oral baits to possums.

**Table 2. Lymphocyte proliferation responses to bovine PPD following vaccination of possums**

| Vaccine group  | Median lymphocyte proliferation (LP) responses to bovine PPD |                       |                      |
|----------------|--|-----------------------|----------------------|
|                | Weeks after vaccination                                      |                       |                      |
|                | 0  | 6                     | 8                    |
| BCG            | 1.7*<br>(0.6, 3.0)   | 54.1<br>(14.4, 92.4)  | 24.7<br>(4.0, 41.4)  |
| WAg539         | 1.6<br>(0.4, 2.5)  | 96.8<br>(13.4, 371.6) | 32.2<br>(13.3, 89.3) |
| WAg563         | 1.0<br>(0.7, 1.2)  | 45.6<br>(4.9, 57.1)   | 19.2<br>(4.0, 28.1)  |
| WAg771         | 1.4<br>(0.2, 3.1)  | 26.1<br>(0.9, 130.8)  | 50.4<br>(3.6, 72.8)  |
| Non-vaccinated | 1.0<br>(0.7, 2.0)  | 2.7<br>(0.6, 5.6)     | 2.4<br>(1.1, 3.8)    |

\* Median LP (range) expressed as a stimulation index (LP response to bovine PPD/LP response with no antigen)

## 4 Evaluation of the efficacy of oral bait BCG vaccine for wild possums

Vaccination studies in caged possums at AgResearch have shown that BCG vaccine delivered orally can induce a significant level of protection against bovine tuberculosis. The next step was to determine whether this vaccine could protect wild possums against natural exposure to *M. bovis*. In collaboration with Landcare Research and Otago University, a field trial to set up in the Orongorongo Valley, near Wellington to test the efficacy of the orally delivered BCG vaccine in wild possums. AgResearch provided key assistance with the trapping, bleeding, vaccinating and necropsy of the possums as well as the monitoring immune responses and funding for the AgResearch component of the trial was provided by Biosecurity NZ.

Two sites were selected in the Orongorongo Valley (3 km apart), with 150 cage traps set at 30 metres apart at each site. The trial commenced in July 2004 and finished in November 2006. The traps were set every 2 months for 4 consecutive nights and all captured possums were initially ear tagged, examined for clinical signs of tuberculosis, blood samples to monitor immune responses to *M. bovis* and released. In November 2004, matched pairs of possums were identified from each site (matched on trap location within the grid, sex and age) with one animal from each matched pair orally dosed with 1 ml of vaccine paste containing BCG vaccine and the other animal serving as a control. Vaccinated possums re-captured in May and November 2005 and May 2006 were revaccinated in a similar manner as before. In addition, new control and vaccinated animals were designated at sites in May and November 2005 to maintain sample sizes. Control possums were lost from the sites at a faster rate than vaccinates and additional control possums were added as well. In total, the trial involved 130 non-vaccinated and 104 BCG-vaccinated possums. The possums were caught in cage traps every 2 months, examined for the presence of tuberculous lesions and then released. Peripheral blood cellular immune responses to *M. bovis* antigens (bovine PPD) and to two specific proteins expressed by virulent *M. bovis*, but not by BCG were monitored every two months to ensure that cases of tuberculosis were not missed just by examining for clinical signs of tuberculosis. At the end of the 2-year study, all the possums were killed and examined for tuberculosis. Samples from any suspect lesions or from pools of lymph nodes from any possum without lesions were collected for bacterial culture.

Oral vaccination with BCG was very effective in preventing possums becoming infected with tuberculosis, with only one tuberculosis-infected possum found in the BCG-vaccinated group and 12 tuberculosis-infected possums confirmed in the non-vaccinated group. Eight incident cases of culture confirmed tuberculosis infection were detected in the 2-monthly samplings, all in the non-vaccinated group and an additional five cases in the final trap-out. All these possums had been at the site for 6 months or more before detection of any lesions, indicating that most, if not all, infections must have occurred on site. *M. bovis* was only isolated from suspect tuberculous lesions and not from any of the samples of pooled lymph nodes. Interestingly, all except one case of tuberculosis was found on one of the sites. An effort was made to increase the force of infection on the other site by the releasing possums that had been experimentally infected with *M. bovis* by the intraconjunctival route, but this did not result in any cross-infection. Possums with culture-confirmed palpable tuberculous lesions generally died within 4 months, although there were some exceptions, with one infected possum surviving for 2 years. The trial has provided evidence that vaccination can prevent tuberculosis infection in a field setting and could be a useful control strategy. Additional information has been obtained on epidemiology of tuberculosis in wild possums.

## 5 Publications in peer-reviewed scientific journals associated with this research contract (July 2006 – June 2007)

Parlane, N.A., Denis, M., Severn, W.B., Skinner, M.A., Painter, G.F., La Flamme, A.C., Ainge, G.D., Buddle, B.M. 2006. Purified and synthetic phosphatidylinositol mannosides are efficient mucosal adjuvants. *Immunological Investigations*, In revision,

Cross, M.L., Buddle, B.M., Aldwell, F.E. 2006. The potential of oral vaccines for disease control in wildlife species. *Vet J.* (in press).

Ainge, G.D., Parlane, N.A., Denis, M., Harer, A., Hayman, C.M., Larsen, D.S., Painter, G.F. 2007. Phosphatidylinositol Mannoside Ether Analogues: Synthesis and Interleukin-12 (IL-12) inducing Properties. *J. Org. Chem.* (in press).

de Lisle, G.W., Buddle, B.M., Collins, D.M. 2007. Bovine tuberculosis – another view from down under. *Vet. J.* 173, 233-234.

Dyer B., Jones, J.D., Ainge, G.D., Denis, M., Larsen, D.S., Painter, G.F. 2007. Total synthesis and structure of native phosphatidylinositol dimannosides. *J. Org. Chem.* 72:3282-3288, 2007.

Collins, D.M., de Lisle, G.W., Aldwell, F.E., Buddle, B.M. A new attenuated *Mycobacterium bovis* vaccine protects brushtail possums (*Trichosurus vulpecula*) against experimental tuberculosis infection. *Vaccine* 25: 4659-4664.

Buddle, B.M., Aldwell, F.E., Keen, D.L., Parlane, N.A., Hamel, K.L., de Lisle, G.W. 2006. Oral vaccination of brushtail possums with BCG: investigation into factors that influence vaccine efficacy and determination of duration of immunity. *N.Z. Vet J.* 54, 224-230.

Aldwell, F.E., Cross, M.L., Fitzpatrick, C.E., Lambeth, M.R., de Lisle, G.W., Buddle, B.M. 2006. Oral delivery of lipid-encapsulated *Mycobacterium bovis* BCG extends survival of the bacillus *in vivo* and induces a long-term protective immune response against tuberculosis. *Vaccine* 24, 2071-2078.

Ainge GD, Parlane NA, Denis M, Larsen DS, Painter GF. 2006. Phosphatidylinositol mannosides: synthesis and adjuvant properties of phosphatidylinositol di- and tetramannosides. *Bioorg. Med. Chem.*, 14, 7615-7624.