



**Australian Government**  
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# Assessment of a DNA Vaccine Encoding *Burkholderia pseudomallei* Bacterioferritin

*Jane McAllister, Penny Gauci, Sharon Lazzaroni, Jodie Barnes,  
Natkunam Ketheesan and David Proll*

**Human Protection and Performance Division**  
**Defence Science and Technology Organisation**

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## **ABSTRACT**

*Burkholderia pseudomallei* is the causative agent of melioidosis, a disease endemic in Southeast Asia and Northern Australia. The bacteria cause infection via subcutaneous or inhaled routes, resulting in either acute lethal sepsis or chronic and eventually fatal disease. Currently no licensed vaccine is available to provide protection against this pathogen. Intracellular enzymatic proteins of other bacterial species, such as the iron storage protein bacterioferritin, have been shown to be potent inducers of the immune response. In this study, a DNA vaccine encoding the *B. pseudomallei* bacterioferritin protein was constructed. The DNA vaccine was then used to immunise mice and analyse subsequent immune responses and protective capability following live challenge with *B. pseudomallei*. There was a substantial increase in anti-bacterioferritin IgG titers following immunisation, however the cellular response and survival following challenge was limited, suggesting that the vaccine may need to be used in conjunction with adjuvant such as CpG or in a multicomponent vaccine in order to increase protective capabilities.

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## Executive Summary

ADF personnel are at risk of being exposed to biological warfare (BW) agents deliberately released by an enemy, and to agents naturally occurring in environments where they are deployed. One such agent is *Burkholderia pseudomallei*, an intracellular bacterium that is the causative agent of melioidosis, a disease endemic in Southeast Asia and Northern Australia. The bacteria cause infection via subcutaneous or inhaled routes, resulting in either acute lethal sepsis or chronic and eventually fatal disease. Currently there is no licenced vaccine to protect against infection with this bacteria.

Recently, scientific advances have seen the use of recombinant DNA techniques to produce DNA vaccines. DNA vaccines act by providing the host with a set of instructions for the synthesis of an encoded bacterial or viral protein. The host cell utilises this information to synthesise the protein, which interacts with the host immune system to generate the immune response and provide protection against disease.

This report describes the construction and assessment of a DNA vaccine against *B. pseudomallei*. The vaccine encodes bacterioferritin, an iron storage protein that has been shown to evoke a strong cellular immune response when encoded in DNA vaccines targeting other bacterial species. Following construction of the vaccine, it was introduced into a mammalian cell culture system to see if the encoded protein could be synthesised *in vitro*. Once it had been demonstrated that the construct contained all the necessary components to produce the protein in mammalian cells, it was then used to vaccinate mice to determine the potential to evoke cellular and antibody based immune responses, and also to assess levels of protection when mice were given a live dose of *B. pseudomallei*. Immunisation with the vaccine did result in substantial production of bacterioferritin-specific antibody, however cellular immune responses and protective responses to live infection were quite low, indicating that as a stand-alone vaccine candidate, bacterioferritin may not be the best choice to use in development of a DNA vaccine against *B. pseudomallei*. It may need to be used in conjunction with adjuvants, or in combination with other DNA vaccine constructs in order to provide protective immunity.

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*Jane McAllister, joined HPPD in 2003. Prior to DSTO, Jane worked at the University of Canberra, identifying potential antigens for vaccines against respiratory pathogens, and assessing their effect on the immune response. Her work in DSTO focuses on the construction and immunological analysis of DNA vaccines against biological agents.*

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### **Penny Gauci**

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*Penny Gauci graduated from the University of Melbourne with a B Sc in 1992. She joined the Department of Applied Biology and Biotechnology, RMIT University as a Technical Assistant in 1992, before being promoted to Technical Officer in 1993. Her work at RMIT included production and purification of polyclonal and monoclonal antibodies, antigen purification, chromatography, vaccine production and animal trials for vaccine efficacy testing. She joined DSTO in 1998 and worked predominantly on the production of antibodies for use in ion channel switch (ICS) biosensors. She joined the DNA vaccines program in 2001, and began work on the production of DNA vaccines for Burkholderia pseudomallei.*

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*David Proll graduated from Monash University in 1993 with a B.Sc(Hons) and went on to complete his PhD in the department of Microbiology. His PhD studies focused on the replication of positive strand RNA viruses. After graduating from university he worked at the Eijkman Institute of Molecular Biology in Jakarta, Indonesia. Here, he investigated the application and development of DNA based vaccines against the parasite that causes Malaria. Upon returning to Australia in 2000, he was recruited by DSTO to initiate a research program investigating DNA vaccines for defence applications.*

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# 1. Introduction

DNA vaccines are a new generation vaccine technology that have the potential to prevent various infectious diseases and have several advantages over conventional vaccine strategies. They pose significantly less risk of side effects than live attenuated vaccines, are cheap to manufacture and have no reliance upon cold chain (1), and can evoke strong cytotoxic lymphocyte (CTL) responses, which are often lacking in strategies such as recombinant protein vaccination (2).

*Burkholderia pseudomallei* is the causative agent of melioidosis, a potentially fatal disease endemic in sub-tropical areas of South East Asia and Northern Australia, including areas such as Darwin and Townsville, where military exercises are conducted. It is also considered a potential biowarfare agent, due to high pathogenicity, ease of aerosolisation, and the prolonged therapy required to treat subsequent disease. It is therefore of interest to the military to develop an effective vaccine against this pathogen. Due to the intracellular nature of the bacteria, an effective vaccine would have to evoke not only an antibody response but also a strong cellular based response. A DNA vaccination strategy would provide a means of activating both the humoral and cellular arms of the immune system (3-5).

Bacterioferritin is an iron storage protein found in a wide range of bacterial species. It has previously been demonstrated that the bacterioferritin gene from *Brucella abortus*, when delivered to mice as a DNA vaccine, evokes a potent Th1 immune response, including strong IFN- $\gamma$  production (6). It is therefore hypothesised that the bacterioferritin gene from *B. pseudomallei* would be an effective vaccine candidate in a DNA vaccine to protect against melioidosis.

The following report describes the construction of a DNA vaccine encoding the *B. pseudomallei* bacterioferritin gene, and subsequent analysis of its efficacy as a vaccine to evoke an immune response and protect against infection in a mouse model. Survival and bacterial clearance from the spleen were assessed following challenge with live *B. pseudomallei*, and humoral and cellular responses were investigated through quantitation of antigen-specific IgG titres, assessment of delayed type hypersensitivity responses and analysis of lymphoproliferation assays.

## 2. Materials and Methods

### 2.1 Bacterial strains and plasmids

Genomic DNA was extracted from *Burkholderia pseudomallei* 08 strains obtained from James Cook University. The pGem T-easy vector was purchased from Promega and the pcDNA3.1 V5-His vector was purchased from Invitrogen. The general laboratory strains of *E. coli* used in this study included TOPO Top10F' and DH5 $\alpha$  (Stratagene). Bacterial cultures were grown overnight in LB broth (Sigma) at 37°C with vigorous shaking. Where appropriate, cultures were supplemented with 50  $\mu$ g /ml ampicillin.

## 2.2 Polymerase Chain Reaction (PCR)

The PCR mixture contained 10 ng genomic DNA template, 40 pmol of both the sense (5' ACGGCTCTAGACAAGGCGACAAGAAAGTCA 3', termed Ag9 start) and antisense (5' AGAGGTCTAGATTCCGGCGAGCCCATCATC 3', termed Ag9 end) oligonucleotide primers (synthesised by GeneWorks), 2x Failsafe™ Buffer F, 1.25U Failsafe™ enzyme in a total volume of 25 µl. The thermal cycling parameters, performed using a Peltier Thermal Cycler-200 (MJ Research), were as follows: 94°C for 5 minutes, 50°C for 30 seconds, 72°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 15 seconds, 72°C for 90 seconds. A final cycle of 94°C for 30 seconds, 55°C for 15 seconds and an extension of 72°C for 5 minutes concluded the thermal cycling. PCR generated DNA was visualised by UV light after electrophoresis through a 1% w/v agarose gel in TAE (40mM Tris-Acetate, 1mM EDTA) containing 0.5 µg/ml ethidium bromide.

## 2.3 Recombinant DNA techniques

DNA ligations, restriction endonuclease digestion and agarose gel electrophoresis were performed according to standard techniques (7). Enzymes were purchased from Promega. DNA was purified from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen) as per manufacturer's recommendations. Following ligation of DNA fragments, plasmid DNA was introduced into TOP10F *E. coli* cells (Invitrogen), made competent by calcium chloride, using the heat shock method.

## 2.4 Plasmid DNA preparation

Small-scale plasmid DNA preparations were extracted from 1.5 ml of a bacterial culture that had been grown overnight in LB broth (Sigma) supplemented with 50 µg /ml ampicillin. Plasmid DNA was extracted from the bacterial cells using the Promega Wizard plasmid purification kit as per manufacturer's recommendations.

## 2.5 Transfection of 293 cells

Six well plates were seeded with  $1 \times 10^6$  Human Embryonic Kidney (HEK) 293S cells per well in 2 ml of growth medium (5% Bovine Calf Serum (ThermoTrace) / SF-DMEM (Gibco - Life Technologies) / 50 µg /ml penicillin-streptomycin) and incubated overnight at 37°C, 5% CO<sub>2</sub>. Two micrograms of plasmid DNA in 250 µl SF-DMEM (Gibco) per well to be transfected, was added to 10 µl of Lipofectamine 2000 (Invitrogen) in 250 µl of SF-DMEM that had been pre-incubated for 5 mins at room temperature. The mixture was then incubated for 20 minutes at room temperature. The cells were washed with 1 ml SF-DMEM, and 2 ml of SF-DMEM added to cover the cell monolayer. The plasmid DNA- Lipofectamine 2000 mixture was added to the appropriate well containing the cell monolayer in 2 ml of SF-DMEM and the plate incubated for 6 hrs at 37°C, in an atmosphere containing 5% CO<sub>2</sub>. The 2 ml of SF-DMEM was removed and replaced with 2 ml of the growth medium. The cells were then incubated for a further 24 hrs at 37°C, 5% CO<sub>2</sub>.

After the addition of 1ml of PBS, the cells were scraped from the plate using a spatula and transferred into a microfuge tube. The cells were washed two times by the addition of 500  $\mu$ l of PBS followed by centrifugation at 200 g for 5 minutes to pellet the cells. The final cell pellet was resuspended in 100  $\mu$ l of lysis buffer and incubated on ice for 15 minutes. The solution was then passed through a 22-gauge needle 10 times to shear the DNA.

## 2.6 Polyacrylamide gel electrophoresis

The harvested cells were prepared for PAGE by resuspending them in an equal volume of 2x Laemmli sample buffer (Sigma) (12.2mM Tris-HCl pH 6.8, 20% Glycerol v/v, 4% SDS w/v, 10%  $\beta$ -Mercaptoethanol v/v, 0.004% Bromophenol Blue w/v) and the sample heated to 100°C for 5 mins, before loading the cell extracts onto a polyacrylamide gel. The prepared samples were separated by electrophoresis through a 12.5% homogeneous SDS polyacrylamide PhastGel (Pharmacia Biotech) as per manufacturer's instructions. Low range, pre-stained, molecular weight standards (BioRad) were also loaded onto the polyacrylamide gel to enable molecular weight comparisons. Following electrophoresis, gels were either stained with Coomassie Blue (Sigma) or electrophoretically transferred to a nitrocellulose membrane (BioRad) for western blotting using the PhastSystem (Pharmacia Biotech) as per manufacturer's instructions.

## 2.7 Western blot analysis

Western blot analysis was performed using standard techniques. In brief, following blocking of the membrane in 10 ml of blocking buffer (5% milk powder in PBS -Tween 20 [Sigma]) for 1hr at room temperature the membrane was washed twice in 20 ml of PBS-Tween 20. The membrane was then incubated in 5 ml of blocking buffer containing Anti-V5 antibody (Invitrogen) at a dilution of 1:5000, for 2 hrs at room temperature, with shaking. The membrane was washed twice in 20 ml of PBS-Tween 20 and incubated in 15 ml of blocking buffer containing goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) at a dilution of 1:30000 for 1hr at room temperature. Following washing of the membrane twice in 20 ml of PBS-Tween 20, proteins recognised by the Anti-V5 antibody were visualised by developing the blot onto x-ray film using ECL and Western Blotting Detection kit as per manufacturer's instructions (Amersham Biosciences).

## 2.8 Preparation of DNA microcarriers for gene gun immunisation

The preparation of the microcarriers was performed as described in the Biorad Helios Gene Gun System instruction manual using a microcarrier loading quantity (MLQ) of 0.5 mg/shot of gold and DNA loading ratio (DLR) of 1  $\mu$ g/shot of plasmid. In brief, 100  $\mu$ l of 0.05M spermidine was added to the required amount of gold and vortexed. The designated concentration of plasmid DNA was then added, followed by the dropwise addition of 100  $\mu$ l 1M  $\text{CaCl}_2$  while vortexing. The mixture was allowed to precipitate at room temperature for 10 min, then spun to pellet the gold, and the supernatant discarded. The pellet was then washed three times with 100% ethanol, before resuspension in ethanol containing the appropriate concentration of polyvinylpyrrolidone (PVP). The gold/plasmid suspension was then coated onto tubing, and 0.5 inch cartridges prepared.

## 2.9 Immunisation of mice

Groups of 20 Balb/c mice were intradermally (ID) immunised on day 0, day 14 and day 28 with either the DNA construct pBps190 or the pcDNA3.1start vector (8) that did not contain the bacterioferritin gene. Ten mice from each group were identified for monitoring survival, 3 for calculation of bacterial loads and 7 to determine the delayed type hypersensitivity (DTH) in the footpad. ID immunisation was achieved using the Helios gene gun (Biorad), which delivered 2 µg DNA at 300 psi into the shaved abdomen of each mouse. Sera were collected from all mice via tail bleed prior to initial immunisation, and subsequently on day 41. On day 42 animals in the survival/bacterial load studies were challenged with live *B. pseudomallei* NCTC 13179 as detailed below.

## 2.10 Challenge with *Burkholderia pseudomallei*

*B. pseudomallei* NCTC 13179, a low virulent strain, was plated onto blood agar 48 hours prior to challenge and incubated at 37°C. A dose of  $9.4 \times 10^5$  cfu (approximately 2 xLD<sub>50</sub>) as described by (9), was used to challenge the vaccinated mice. A suspension of  $1 \times 10^8$  was made up in sterile PBS with an optical density reading at 650 nm of 0.198. The dose was then diluted to achieve the required concentrations in 200 µl and the suspension was then placed into syringes. The mice were infected with the bacteria via intraperitoneal inoculation and monitored for 10 days for survival and any moribund mice were euthanised using CO<sub>2</sub> asphyxiation.

To determine the precise inoculum dose for the mice, ten-fold serial dilutions were made in PBS and 20 µl of each dilution were plated in triplicate onto Ashdown's agar and incubated at 37°C for 24-48 hours. The colonies were counted and the dose was calculated.

## 2.11 Bacterial loads

Bacterial load measurements were carried out on 3 mice from both groups (sham and pBps190) at day three. Mice were euthanised via CO<sub>2</sub> asphyxiation. Spleens were removed aseptically and placed into a stomacher bag with PBS. The bags were then placed into a stomacher until the spleens were homogenised. Ten-fold dilutions were made from the homogenate and 20 µl aliquots plated onto Ashdown's agar in triplicate and incubated for 24-48 hours. The colonies were counted and the concentration of bacteria in the whole spleen calculated.

## 2.12 Analysis of serum IgG by ELISA

ELISA were performed in 96 well plates using standard methods. For each microtitre plate used to assay the levels of antigen-specific IgG, a standard curve was included (see Appendix). This consisted of serial dilutions in bicarbonate coating buffer of known concentrations of mouse IgG (5 mg/ml; Sigma) as listed in Appendix A1. Briefly, the wells of the microtitre plate were coated with either 1 µg of recombinant bacterioferritin or the mouse IgG standard in 100 µl of bicarbonate buffer (0.1M Na<sub>2</sub>CO<sub>3</sub>, pH 9.5), and incubated overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20, and blocked

with 3% skim milk in PBS/Tween at RT for 1 hr. The plate was washed, serum samples diluted in 3% skim milk/PBS/Tween, and 100 µl added in triplicate to the appropriate wells. The 3% skim milk/PBS/Tween was added to standard wells. The plate was incubated at 37°C for 20 mins, followed by incubation at RT for 40 mins with agitation. After washing three times as above, 100 µl of anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1:2000 in PBS, was added to each well. The plate was incubated at 37°C for 30 min followed by 30 min at RT with gentle agitation. The plate was washed, and 100 µl of *p*-nitrophenyl phosphate substrate (Sigma) added to each well. The colour was allowed to develop for 1 hr, before the plate was read on a microplate reader at 405 nm.

## 2.13 Lymphoproliferation Assay

Spleens were disrupted in a petrie dish with 1-2 ml RPMI culture medium (5% fetal calf serum, 2mM L-glutamine, 1M HEPES, 5x10<sup>-5</sup>M 2-β-mercaptoethanol, 1% penicillin/streptomycin) using a 2 ml syringe plunger to extract the lymphocytes. The cell suspension was transferred to a 10 ml centrifuge tube and cells pelleted at 100xg for 5 mins. The pellet was resuspended in 500 µl red blood cell (RBC) lysing buffer (0.17 M NH<sub>4</sub>Cl, pH 7.2-7.4, sterile filter) for 1min before dilution in RPMI culture medium and centrifugation as before.

The supernatant was removed and the pellet washed twice in RPMI culture medium by centrifuging at 100xg for 5 mins. After the final wash the pellet was resuspended in a known volume of RPMI culture medium and viable cells counted by Trypan Blue exclusion. Briefly, 50 µl of cells were added to an Eppendorf tube containing 200 µl of PBS and 250 µl of Trypan blue (Sigma), and mixed thoroughly. The viable lymphocytes (those excluding the trypan blue) were counted using a Neubauer haemocytometer. The number of cells/ml was determined using the following equation:

cells/ml = number of cells in middle square of haemocytometer x dilution factor x 10<sup>4</sup>

Cell volume was then adjusted with RPMI culture medium to give a final concentration of 2x10<sup>6</sup> cells/ml.

One hundred microlitres of cells, at a concentration of 2x10<sup>6</sup> cells/ml, were added in triplicate to the flat-bottom wells of a 96 well microculture plate (Nunc). Recombinant bacterioferritin suspended in RPMI culture medium was added to the cells to give a final concentration in culture of 5 µg/ml. Cells acting as negative controls received 100 µl of RPMI culture medium alone while cells acting as positive controls received 100 µl of the mitogen Concanavalin A (Con A) (5 mg/ml; Sigma), suspended in RPMI culture medium to give a final concentration 5 µg/ml in culture.

On day 4 of cell culture, cells were pulsed by adding 0.5 µCi (25 µl of a 1/50 dilution in RPMI culture medium) of methyl-<sup>3</sup>[H] thymidine (Amersham Biosciences Pty Ltd) to each well of the 96 well microtitre plate. 16 hours after pulsing, cells were harvested onto filter mats and left overnight to dry. The following day the mats were coated in 5 ml of scintillation fluid according to the manufacturer's instructions (Betaplate Scint, Wallac) and proliferation assessed by counting for 1min on a Betaplate scintillation counter.

## 2.14 Delayed Type Hypersensitivity (DTH) responses

DTH responses were assessed by subcutaneous injection of 5 µg of *B. pseudomallei* lysate into the left hind footpad. An equal volume of PBS was injected subcutaneously into the right hind footpad. Thicknesses of left and right footpads were measured with digital calipers at 0, 24 and 48 hrs post-footpad challenge. Change in footpad thickness was calculated by subtracting mean measurements (n=5 per foot) at 0 hrs from footpad measurements at 24 and 48 hrs after challenge.

# 3. Results and Discussion

## 3.1 Construction of the DNA Vaccine

The genetic sequence of the bacterioferritin protein from *B. abortus* was used to identify a homologous sequence in the *B. pseudomallei* K96243 genome, using BLAST (10) a computer based program for identifying homologous DNA sequences. The identified DNA sequence was utilised for primer design, and subsequently the primers used to amplify the bacterioferritin gene in genomic DNA extracted from the 08 strain of *B. pseudomallei*. The resulting PCR product was visualised on a 1% agarose gel, then purified for ligation into pcrII TOPO intermediate vector. Following cloning of the bacterioferritin gene product into pcrII TOPO to form the intermediate construct pBps090, the gene was excised from pcrII TOPO by digestion with Xba I and re-ligated into the mammalian expression vector pcDNA3.1start, to form the DNA vaccine construct pBps190 (Figure 1).

In order to assess the integrity of the constructed DNA vaccine, pBps190 was transfected into the mammalian cell line HEK 293S. Twenty-four hours after transfection, the cells were harvested and lysed. The cell extracts were then visualised by PAGE and the proteins transferred onto nitrocellulose for immunoblotting. The immobilised proteins were probed via western blot, using an anti-V5 antibody specific for the V5 epitope attached to the carboxy terminal of the bacterioferritin protein. The molecular weight estimated for the detected protein correlated with the expected molecular weight of 23.5kDa for the *B. pseudomallei* protein encoded by the pBps190 DNA vaccine (Figure 2). This demonstrated that the engineered vaccine contained all the necessary information to direct protein synthesis of the encoded protein in a mammalian cell system, indicating that when introduced into higher mammals the protein could be expressed *in vivo*.

## 3.2 Immunisation and challenge of mice

Once it was determined that pBps190 could induce expression of bacterioferritin in mammalian cells *in vitro*, the effect of immunising mice with the DNA vaccine, including subsequent protection against *B. pseudomallei* infection was investigated. Two groups of 20 mice were immunised 3 times over a 4 week period (day 0, day 14 and day 28) with 2 µg of either pBps190 or pcDNA3.1start (sham) and were subsequently IP challenged on day 42 with 2xLD50 live *B. pseudomallei* NCTC 13179. Survival was monitored in 10 mice from each group

over a 10 day period. All 10 sham immunised mice had succumbed to challenge by day 3 (Figure 3), however one mouse immunised with the pBps190 survived until the experiment was terminated at 10 days post-challenge. The survival of this mouse is unlikely to be significant, as a repeat trial involving 10 mice vaccinated with Bps190 resulted in all mice succumbing to infection when challenged with live *B. pseudomallei* (unpublished data). An experiment in which pBps190 was used as initial vaccine (2 x vaccination with 2 µg DNA, 21 days apart), to prime the immune response, followed by 3 boost immunisations with a corresponding recombinant protein engineered from *B. pseudomallei*, have also failed to produce a significant protective response against subsequent challenge with the bacterium (unpublished data).

Although there was no significant protection against death caused by infection with *B. pseudomallei* afforded by the DNA based vaccine, analysis was performed to determine the bacterial loads in the spleens of infected animals (Figure 4). The purpose of this was to determine if the DNA vaccine was having an effect, albeit insufficient, to limit the bacterial infection. The bacterial loads in the spleens of 3 of the mice from each group were determined, however 1 mouse from each group succumbed to infection before day 3. Therefore because only two mice per group were assessed, a definitive statement about the ability of vaccinated mice to clear bacteria from the spleen cannot be made, but it is likely that vaccination had no effect on bacterial loads.

In order to determine whether any antigen-induced cellular responses could be observed in vaccinated animals, lymphocytes from the spleens of pBps190 immunised animals were excised and re-exposed *in vitro* to 5 µg/ml recombinant bacterioferritin. There was no difference in bacterioferritin-specific proliferation of lymphocytes when compared to those taken from the spleens of sham immunised mice (Figure 5), indicating that antigen-specific T cell responses were not evoked by the vaccine. Delayed type hypersensitivity was also assessed by measuring the footpad thickness (indicating inflammatory response) of mice following injection of recombinant bacterioferritin, but once again no difference could be observed between Bps190 and sham immunised groups (Figure 6)

### 3.3 Analysis of the humoral immune response

The humoral immune response plays an essential role in the elimination of many bacteria from the host, thereby controlling unwanted infections. Many vaccines, in particular protein or sub unit vaccines, focus on inducing a strong humoral response and thus high antibody titres in order to provide protection against subsequent exposure to the disease target. In order to assess the antibody levels the *B. pseudomallei* bacterioferritin DNA vaccine was inducing, serum samples were collected from each mouse prior to immunisation and on day 41, approximately 2 weeks after the third immunisation, just prior to bacterial challenge. The titres of bacterioferritin-specific IgG for each serum sample were determined by ELISA. Antibody concentrations were determined using a standard curve constructed from known concentrations of mouse IgG (appendix A2). Preimmune titres were subtracted from the day 41 post-vaccination titres in order to determine total bacterioferritin-specific IgG titres. Results indicate that immunisation of mice with the pBps190 DNA plasmid resulted in an increased production of IgG specific to *B. pseudomallei* bacterioferritin (Figure 7). Several mice displayed specific IgG titres of more than 200 µg/ml. The increase in antigen-specific titres induced

through vaccination with pBps190 is considerable when compared to animals immunised with the control vaccine, pcDNA3.1start. This indicates that although limited protection was observed following immunisation with pBps190, the vaccine is capable of inducing a substantial humoral response. It also suggests that antigen-specific antibody alone may not be capable of clearing a *B. pseudomallei* infection, highlighting the important role of cytotoxic T cell immunity in intracellular bacterial infection.

### 3.4 Analysis of cellular response

The lack of a strong cellular response following immunisation with pBps190 may be an indication that bacterioferritin is not a suitable candidate antigen for a vaccine against *Burkholderia pseudomallei*. In contrast to our study, a strong T cell proliferative response was observed following intra-muscular (*im*) immunisation with a DNA vaccine encoding *B. abortus* bacterioferritin (6), demonstrating that the protein can evoke cellular immunity. Perhaps the differing results seen between the two studies can be attributed to method of delivery of vaccine, as it has been shown previously that different delivery methods (eg *im* injection versus gene gun administration) can influence the level and type of immune response (11, 12). It has been demonstrated previously that *im* immunisation tends to result in a predominantly Th1 response, while gene gun immunisation results in a Th2 bias (11-15). This is primarily attributed to the differing quantities of DNA required for each type of immunisation. Intramuscular injections require much higher concentrations of DNA, and one study has shown that by raising the concentration of DNA administered by a gene gun to that of *im* injection, the Th2 bias could be switched to Th1, with antibody isotypes switching from IgG1 to IgG2a (13). Essentially, much of the observed preference of *im* injection to promote Th1 responses is due to adjuvant effects of using large amounts of bacterial plasmid DNA.

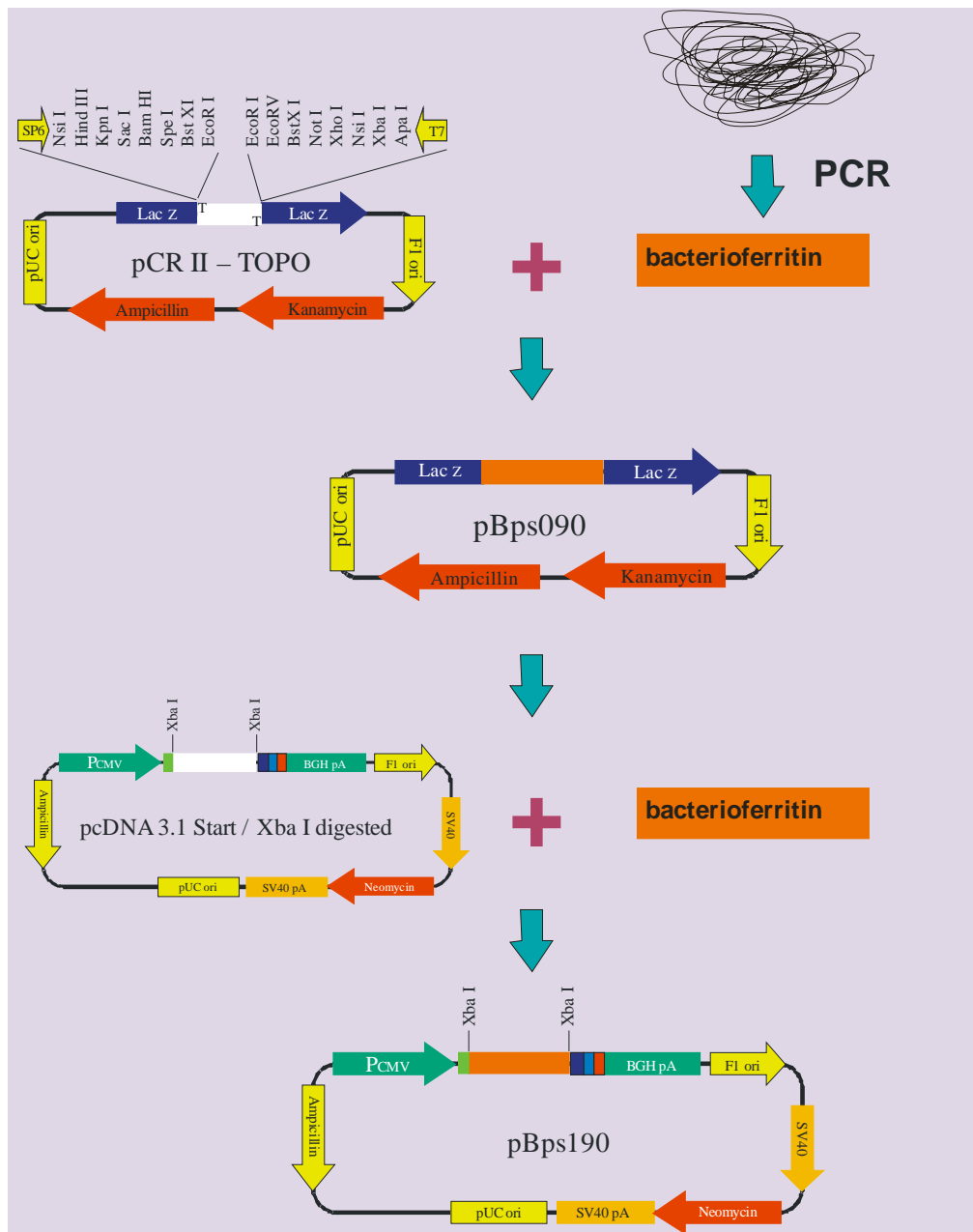
Although DNA vaccination as a general rule promotes Th1 immunity, perhaps in this study it has been overshadowed by the method of vaccine delivery, and by delivering only 2 µg of DNA by gene gun, rather than the 100 µg of DNA that was administered by IM injection in the *B. abortus* study, this has resulted in a predominance of a Th2 microenvironment, which may include a lack of sufficient IFN-γ required to promote cellular immunity.

It is also important to note, that although a strong cellular response was seen using *B. abortus* bacterioferritin, mice still were not protected against intra-peritoneal challenge (6). Because bacterioferritin is a cytosolic protein, it is seen later in the immune response when the bacteria have been broken down, and it is likely that it may not be as immunogenic as the outer membrane and surface proteins that are also used as antigens in vaccine development.

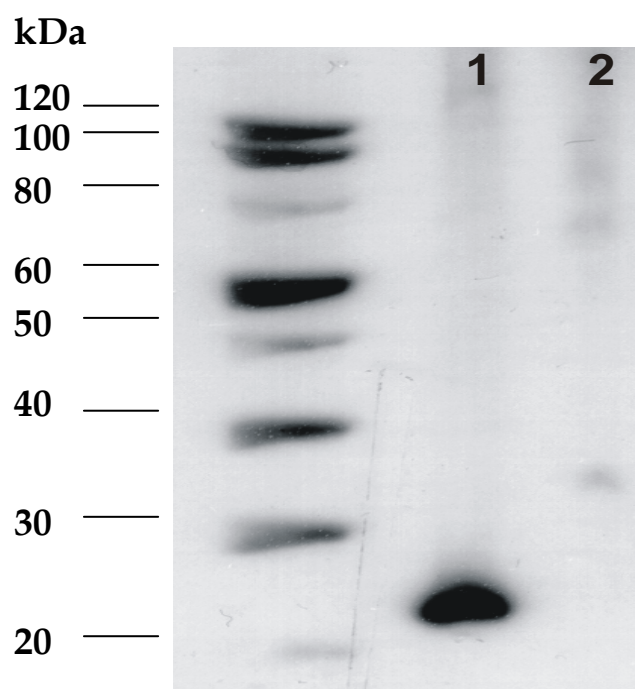
Finally, *B. pseudomallei* is an intracellular pathogen and infection is a complex process. Because disease can manifest as a chronic or acute condition, it is likely that a DNA vaccine encoding a single antigen may not necessarily provide a high level of protection on its own. It may need to be used in conjunction with adjuvants, such as CpG motifs or cytokines, or in combination with other DNA vaccine constructs in order to provide protective immunity.

## 4. Conclusion

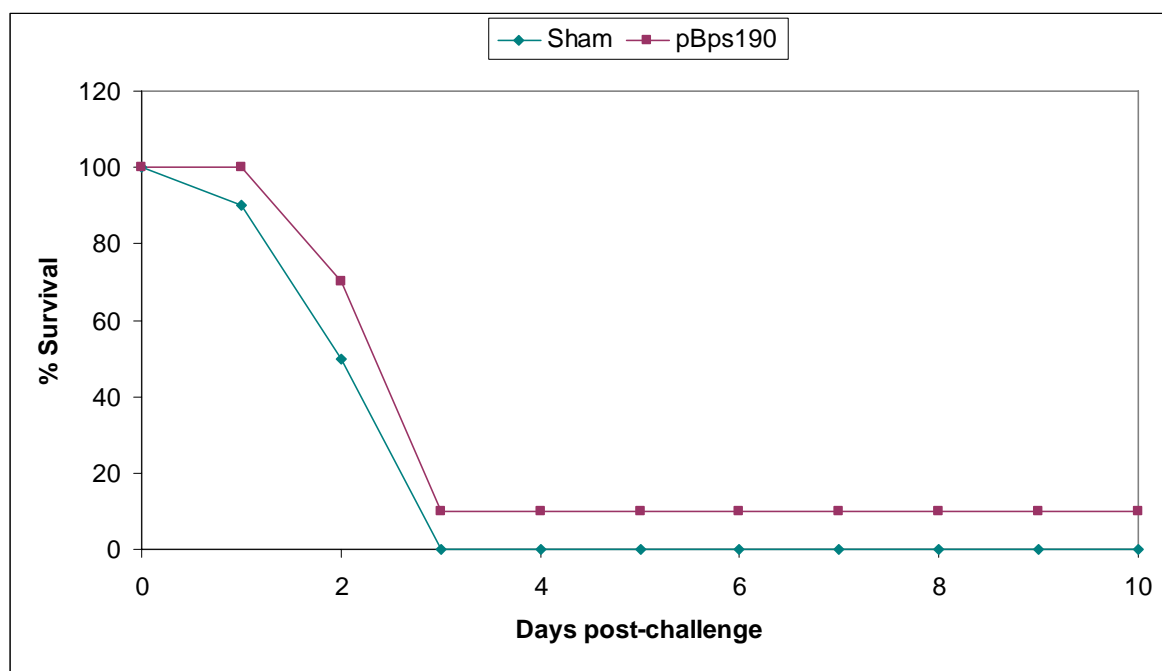
This study has detailed the construction of a DNA vaccine encoding the *B. pseudomallei* bacterioferritin gene. It was shown that the DNA vaccine, while capable of inducing a substantial humoral response, lacked the ability to evoke strong cellular immune responses or protect against infection with the bacterium. Therefore the *B. pseudomallei* bacterioferritin DNA vaccine, is not a suitable vaccine candidate in its own right, rather it may be better suited as a component of a multi-candidate vaccine against *B. pseudomallei*. Further studies will examine the formulation of this vaccine as part of a complex multi-component vaccine.



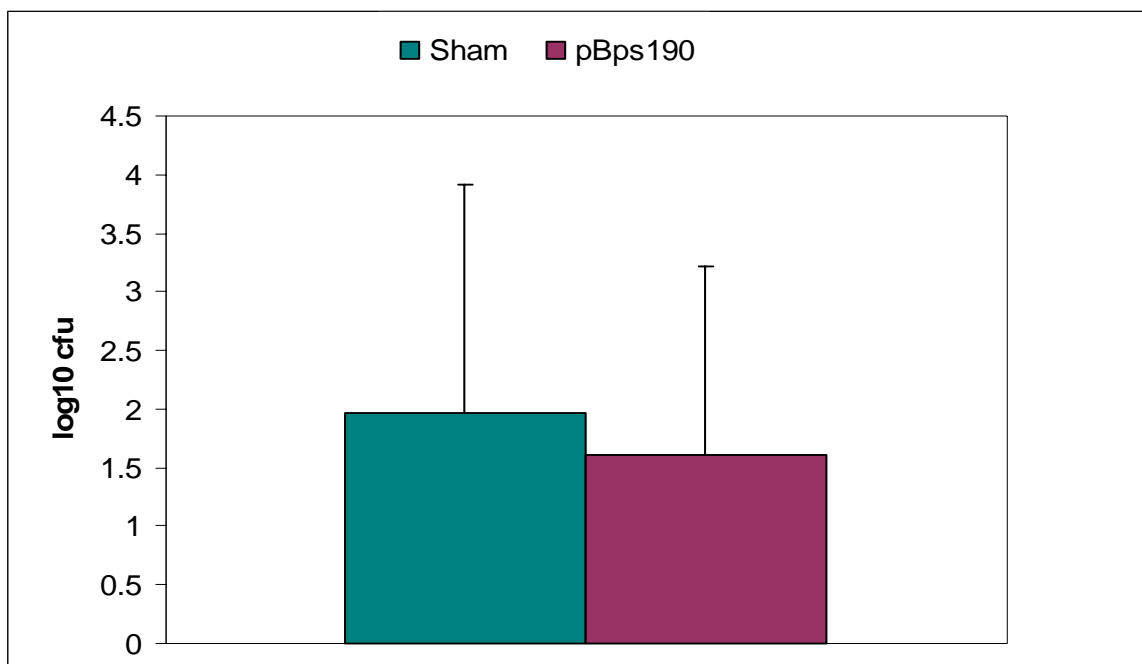
**Figure 1: Construction of the bacterioferritin DNA vaccine Bps190.** The bacterioferritin gene was amplified by PCR and cloned into the intermediate vector pCRII TOPO to ensure alignment and a correct reading frame. This intermediate construct was designated Bps090. The gene was then excised by restriction enzyme digestion with Xba I and subcloned into the mammalian expression vector pcDNA3.1start, to create the DNA vaccine construct Bps190.



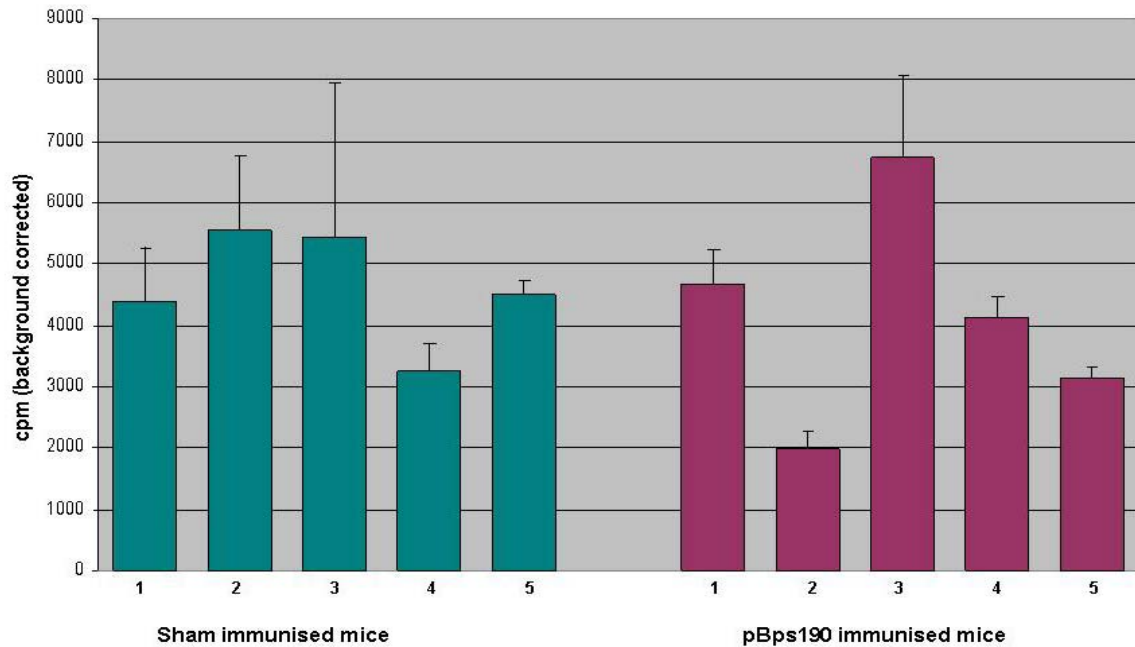
**Figure 2:** *In vitro* expression of bacterioferritin encoded in the DNA vaccine detected by western blot. The pBps190 plasmid construct was transfected into mammalian 293S cells using a cationic lipid complex. Extracts from transfected (lane 1) and non-transfected control (lane 2) cells were resolved by PAGE (12.5%), transferred to a nitrocellulose membrane and probed with anti-V5 epitope antibody. Reactive proteins were visualised through a HRP-conjugated secondary antibody developed by ECL



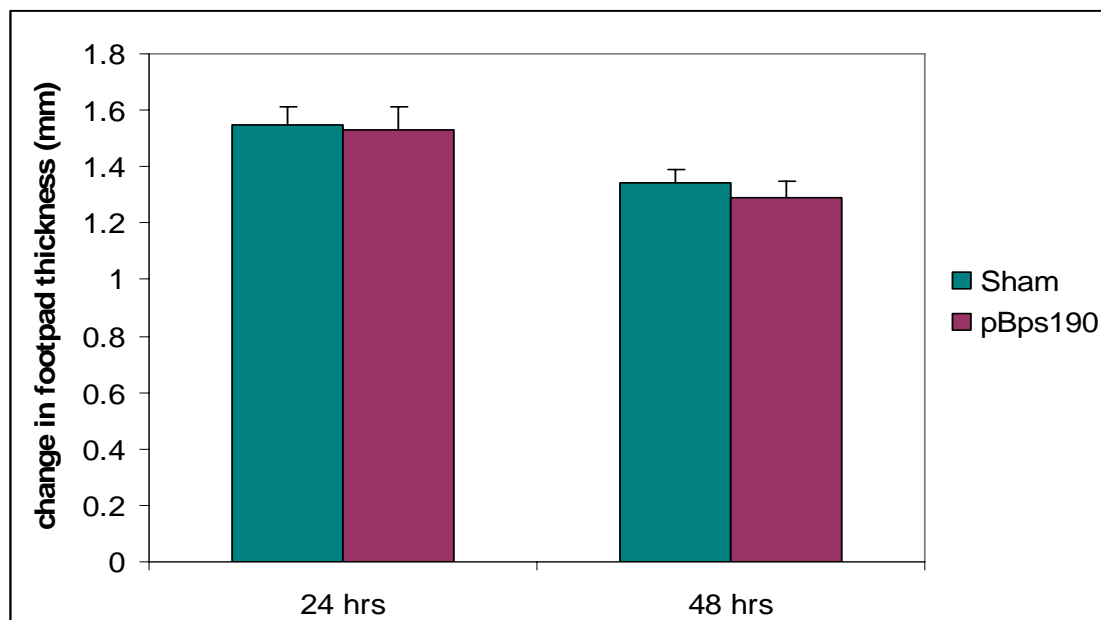
**Figure 3:** *Survival of mice following challenge with live B. pseudomallei* NCTC 13179. Ten mice immunised with either the pBps190 or pcDNA3.1start alone (sham) received 2xLD50 IP of live *B. pseudomallei*, 2 weeks after the third immunisation. Survival was monitored over a 10 day period post-challenge.



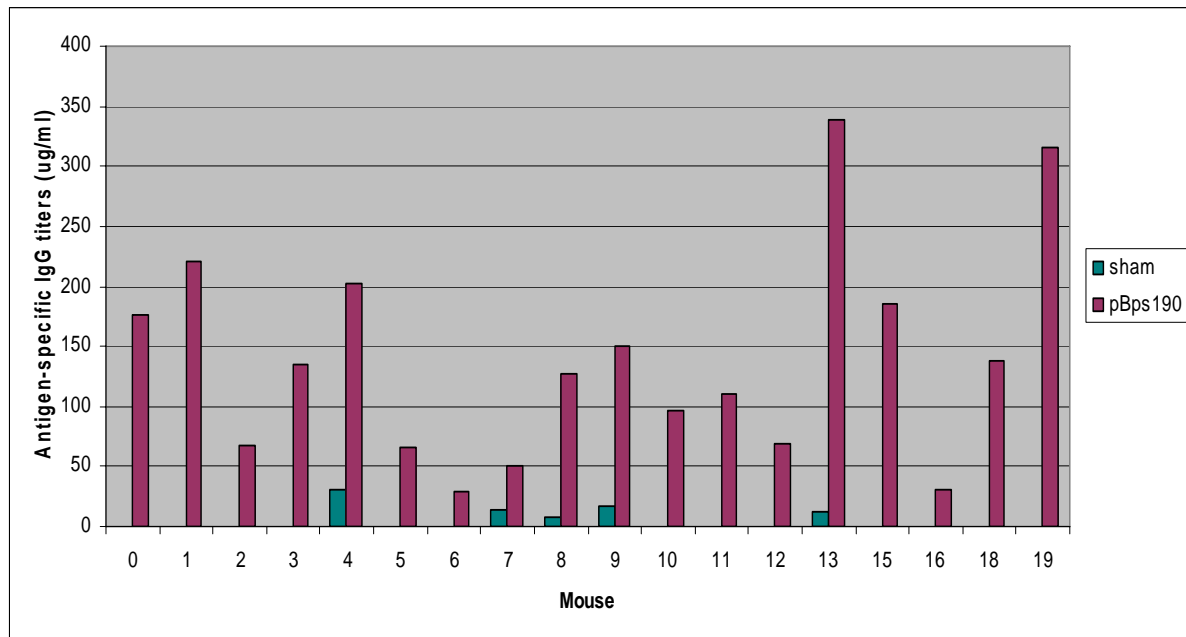
**Figure 4:** *Bacterial loads in spleens of mice following DNA vaccination and challenge with live *B. pseudomallei* NCTC 13179. 3 mice from each immunisation group (pBps190 and sham) received 2xLD50 of live *B. pseudomallei* NCTC 13179 IP, 2 weeks after the third DNA vaccination. Three days after challenge animals were sacrificed and the spleens homogenised. Ten-fold dilutions were made from the homogenate and plated onto Ashdown's agar in triplicate in 20µl aliquots and incubated for 24-48 hours. The colonies were counted and the concentration of bacteria in the whole spleen calculated. Values are expressed as the mean±SD*



**Figure 5:** *Antigen-specific proliferation of lymphocytes from the spleens of pBps190 and sham immunised mice.* Lymphocytes were extracted from the spleens of immunised animals and exposed to 5  $\mu\text{g/ml}$  of recombinant bacterioferritin *in vitro*. On day 4 of culture cells were pulsed by adding 0.5  $\mu\text{Ci}$  of methyl- $^3\text{[H]}$  thymidine and incubated for a further 18 hours. Cells were then harvested onto filter mats and proliferation counts determined using a Betaplate scintillation counter. Background counts (cells exposed to media only) were subtracted from sample counts. Values are displayed as average cpm  $\pm$  SEM



**Figure 6:** *Delayed Type Hypersensitivity (DTH) response in footpad of mice.* Mice that had received 3 vaccinations of either pBps190 or pcDNA3.1start (sham) were injected with 5 ug of *B. pseudomallei* lysate into the footpad and changes in the thickness of the footpad (indicating an inflammatory response) measured at 24 hr and 48 hr. Values are expressed as the mean change in footpad thickness of each immunisation group  $\pm$  SEM



**Figure 7: ELISA analysis of the humoral immune response to *B. pseudomallei* bacterioferritin.** Antigen-specific IgG titres were measured in sera taken from mice vaccinated with the pBps190 construct or with the pcDNA3.1start vector alone. Animals were intra-dermally administered a 2 µg dosage of DNA on days 0, 14 and 28. Sera were collected from each mouse pre-immunisation and on day 41, approximately 2 weeks after the third immunisation. Bacterioferritin-specific IgG titres were then measured by ELISA. Values are expressed as the mean post-immunisation titres minus the mean pre-immune titres, with all samples measured in triplicate. NOTE: Mice 14 and 17 died prior to post-immunisation sera collection on day 41.

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19. ABSTRACT <i>Burkholderia pseudomallei</i> is the causative agent of melioidosis, a disease endemic in Southeast Asia and Northern Australia. The bacteria cause infection via subcutaneous or inhaled routes, resulting in either acute lethal sepsis or chronic and eventually fatal disease. Currently no licensed vaccine is available to provide protection against this pathogen. Intracellular enzymatic proteins of other bacterial species, such as the iron storage protein bacterioferritin, have been shown to be potent inducers of the immune response. In this study, a DNA vaccine encoding the <i>B. pseudomallei</i> bacterioferritin protein was constructed. The DNA vaccine was then used to immunise mice and analyse subsequent immune responses and protective capability following live challenge with <i>B. pseudomallei</i> . There was a substantial increase in anti-bacterioferritin IgG titers following immunisation, however the cellular response and survival following challenge was limited, suggesting that the vaccine may need to be used in conjunction with adjuvant such as CpG or in a multicomponent vaccine in order to increase protective capabilities.					