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Testing and verification of a single-dose cattle tick vaccine

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Abstract

Cattle-ticks are the most important parasite affecting the Australian cattle industry, resulting in annual losses exceeding $160M. Sustainable and effective control of cattle-ticks through vaccination is a well-established approach. However, current cattle-tick vaccines require multiple doses and frequent booster doses to provide cattle with sustained protection. This multi-dose vaccination approach cannot be effectively applied to most of the at risk cattle in northern Australian where cattle are only mustered once or twice a year. To address this issue, this project evaluated a novel single-dose formulation of the Bm86 cattle-tick antigen for its capacity to stimulate immune responses in cattle. The optimal single-dose formulation elicited strong and sustained immune responses in the study cattle, measured by their antibody levels. While these immune responses peaked 28 days after immunisation, they remained at high levels for one year. In a second group of cattle, the capacity of the novel single-dose vaccine to protect cattle from cattle-tick larval infestations 56 days after immunisation was also tested. The efficacy of the single-dose formulation was estimated to be 76.9% in an induced infestation study. This level of protection is equivalent to those reported in multi-dose vaccination studies. The results of this project demonstrate the feasibility of a single-dose cattle-tick vaccine. With further development the single-dose offers the prospect improved and sustainable control of cattle-ticks in northern Australia cattle.

Executive summary

Cattle-ticks are the most important parasite affecting the Australian cattle industry, resulting in annual losses estimated to be $161M. Currently cattle-tick are controlled through the use of chemicals. However there are increasing reports of cattle-ticks developing resistance to the current range of acaricides. Previously, an effective cattle-tick vaccine was available in Australia. Indeed a very similar vaccine is still used for the effective control of cattle-ticks in Cuba and parts of South America. In the context of the northern Australia cattle herd, the most significant drawbacks of the cattle-tick vaccine were that it initially required multiple doses to stimulate protective immune responses in cattle and then frequent booster doses to sustain protection, Whereas these requirements are not an impediment to the vaccine's success in Cuba, it meant the vaccine could not be effectively applied to most of the at risk cattle in northern Australian where cattle are only mustered once or twice a year. To address this issue, the current project had two key objectives; to evaluate a novel single-dose vaccine formulation to test its capacity to stimulate immune responses in cattle and to determine if these immune responses would protect them from experimental infestation with cattle-tick larvae.

The first objective was addressed in a trial to determine if the amount of cattle-tick antigen (Bm86) included in the vaccine formulation had an effect on the immune responses. This was tested using three different amounts (100μg, 200μg and 300μg) of the antigen in groups of cattle (n=10 in each group). Overall, the single-dose formulation elicited strong and sustained immune responses in the study cattle, as measured by their levels of circulating antibody. The amount of antigen did not appear to have a major effect on the immune responses in the cattle in the first eight months of the trial. Across the three groups, the immune responses peaked 28 days after injection, then consistently dropped over the next two months. Then, the immune response increased during the next month and remained reasonably stable until seven months after immunisation. There was a marginal drop in the responses at nine months, again a consistent observation across all the here groups. After this point, the immune responses between the groups began to diverge. The responses in the group immunised with 300μg of Bm86 remained constant, while the responses in the other groups declined. These results suggest that the higher the amount of antigen, the more persistent the immune responses were. These results suggest the 300μg dose would be ideal for the planned protection study. The results described above are based on the average responses observed in each treatment group. Another important consideration in these types of studies is the immune responses of individual animals within each treatment. Overall, the cattle from each group all responded to the initial dose of the single-dose vaccine and broadly speaking followed the pattern of immune responses described above. However, within each group the cattle could be broadly divided into high and low responders. These categories became more evident as the trial progressed and were readily apparent at six months post-immunisation. Again, the group immunised with 300μg of the antigen tended to have fewer animals that might be classified as low responders. These results supported the use of the 300μg dose in the cattle-tick infestation study.

To investigate how the single-dose vaccine might be applied in an industry setting, the responses of cattle to booster immunisations were also examined. After one year, the remaining cattle (n=6) from the 300μg dose treatment were given a booster injection of the complete vaccine formulation. Their circulating levels of antibody to Bm86 increased dramatically and remained high for two months after the booster dose. At three months post-boost the responses in five of the animals began to decrease and continued to decline until the end of the experiment. However, six months after the boost the responses were still higher than those detected just prior to delivery of the boost dose. Swelling was observed at the site of booster-dose injection in these animals. The size of these lumps ranged from large (e.g. soft-ball size) to absent (in one animal) after six months. Despite these reactions, the animals appeared to remain healthy throughout this period with no increased

temperature, loss of appetite or behavioural changes detected. Similarly, the presence, size or absence of a lump at the injection site did not influence the immune response to the vaccine antigen. At the end of the trial, samples of the lumps were submitted for pathological assessment which suggested they were due to an allergy type response to the booster dose of the vaccine. Further research is required to determine if these reactions can be minimised. While they did not appear to adversely affect the health of the cattle in a pen trial environment, under field trial conditions they may be susceptible to further complications.

The capacity of the observed circulating antibodies to protect cattle from induced cattle-tick infestations was also tested. Similar to the initial trial assessment of the immune responses, cattle (n=24) could be categorised as high or low responders. The high responders (n=16) showed an average level of protection of 76.9%, which is higher than recently reported in multi-dose vaccination studies (74%). Importantly, the cattle in this study were not challenged until 56 days after the administration of the single vaccination. To our knowledge this the longest interval between vaccination and challenge of any published cattle-tick vaccine studies. Moreover there are no published studies reporting single-dose formulations for cattle-tick vaccines. The results of this project demonstrate the feasibility of single-dose cattle-tick vaccine. With further development to improve the suitability of the vaccine for industry, the single-dose offers the prospect improved and sustainable control of cattle-ticks in northern Australia cattle.

Research is required to establish if the level of antibodies to Bm86 elicited by immunisation can be correlated to the level of protection observed in cattle infestation studies. This could be achieved by infesting cattle at different time points after immunisation to quantify the level of protection from larval tick infestation observed. This correlation would provide a considerable cost-saving in the further development of the vaccine as it would reduce the need for the very expensive infestation trials and would be crucial in assessing the performance of the vaccine in future field trials. Research is required to determine if the vaccine formulation could be modified to decrease the proportion of immunised cattle nominally classed as low responders. This would provide two direct benefits. Firstly, a greater percentage of the immunised cattle herd would be protected, thus providing a better return on the investment in vaccination for producers. Secondly, more effective vaccination would have a more dramatic effect in reducing the surviving cattle-tick population, thus providing sustainable long-term control of this pest. One way to achieve this may be to test alternative adjuvants in the formulation.

Another research activity would be to determine if the severity of the apparent allergic reaction to the booster dose could be reduced or eliminated. This would require identification of the formulation component(s) which are causing the allergenic type response. This could be achieved by exposing immunised cattle to the individual components to see if similar responses result. Once identified the amount of the component could be reduced or substituted to alleviate the allergenic type response. The risk of this approach is that the effectiveness of the primary immunisation might be reduced as well.

The completed studies clearly support the further development of the single-dose cattle tick vaccine formulation tested in this project, as it shows considerable promise to provide sustainable control of this important pest. The single-dose cattle tick vaccine provides the key advantage of being amenable to use within the constraint of the current industry practice of infrequent mustering. An effective and usable cattle tick vaccine would also reduce industry reliance on chemical control methods. It has been estimated that optimal control of cattle-ticks would return $61M in lost productivity to northern Australian cattle producers. An effective cattle tick vaccine would be a highly desirable addition to the integrated pest-management system required to delivery this return to industry.

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1 Background

The cattle tick (*Rhipicephalus australis*) is the costliest pest of cattle in northern Australia, with an estimated annual impact of $156M (Lane et al., 2015). This impact is made up of blood loss, hide damage, behavioural disturbance and the transmission of tick fever. Cattle tick control over recent years has relied on the more resilient *Bos indicus* genotype and the use of chemicals (either tick growth regulators, which provide up to three months of protection, or more frequent application of short-acting acaricide products). Acaricide-based control is becoming increasingly difficult in many areas because resistance to all families of acaricide, including the growth regulators, has now been recorded in Australia, whereas the indicine genotype suffers the disadvantage of inferior beef eating quality. To address these issues and ensure that producers can continue to effectively control cattle tick infestations it is necessary to develop complementary control strategies to reduce the current reliance on chemicals and facilitate the development of multi-faceted integrated cattle tick control systems. One such strategy is the application of a cattle tick vaccine whereby the immune systems of cattle are harnessed to control infestations. It has been estimated that if all cattle at risk of cattle tick infestation could be reduced to the lowest risk of infestation there would be an annual return of $61 million to industry (Lane et al., 2015).

A cattle tick vaccine, TickGard^{PLUS}, based on the Bm86 antigen, was previously developed and commercialised in Australia (Willadsen et al., 1995). While effective, TickGard^{PLUS} was difficult to utilise in the northern cattle herd as the initial vaccination protocol recommended three doses with frequent booster vaccinations to ensure continued protection. Despite these limitations the vaccine did achieve good market penetration in Australia becoming the highest selling cattle tick pharmaceutical in the first four years it was on the market (de la Fuente et al., 2007). The results of one Australian on-farm study of the impact of the vaccine on acaricide usage reported on average 2.4 times fewer treatments and over 25% of properties completely eliminating treatments (Cobon et al. 1995 as reported by de la Fuente et al. (2007). Furthermore a long-term review of TickGard^{PLUS} and similar products in other countries demonstrated that vaccination was an economically viable method of tick control and minimised the use of chemical residue issues (de la Fuente et al., 2007). However, in Australia the vaccine ultimately failed because of a combination of factors, including the technical requirements for multiple initial injections and frequent booster shots being incompatible with the extensive cattle industry's mustering practices. The cost of mustering in the northern Australian cattle herds makes it prohibitively expensive to alter the frequency of practice just to comply with a multi-dose vaccination regimen. As an example, an MLA study of the Kimberley and Pilbara regions reported that of 71 properties surveyed, 40 and 28 of respondents only mustered once or twice annually (Dray et al. 2011). Furthermore the average cost of mustering ranged from $21.79 to $38.65 per head depending on location (Dray et al. 2011). These costs clearly demonstrate the number and frequency of doses is an important consideration in the development of vaccines for the northern Australia beef industry. Sequential veterinary health company mergers and acquisitions and resulting changes in product focus also contributed to TickGard^{PLUS} being withdrawn from the market. The vaccine continued to be available to the Queensland dairy industry until 2010 with supply of remaining stocks of the vaccine being facilitated by the industry peak body. The continued successful use by the dairy industry suggests vaccination can be an effective method for sustainable control of cattle ticks.

These previous studies and industry experiences suggest that a cattle tick vaccine that could be applied in alignment with current industry practices, such as infrequent mustering, would be a significant aid in improving the control of this parasite. Ideally this vaccine would require a single-dose and provide a minimum of six months protection from infestation. To address this issue the CRC for Polymers in collaboration with The University of Queensland, Virbac (Australia) and the Queensland Government developed novel single-dose vaccine formulations using biopolymers to deliver the cattle tick Bm86 antigen (the active component of TickGard^{PLUS}). Previously, this collaborative team developed biopolymer formulations, through testing in sheep, which stimulated strong and sustained immune responses (measured by circulating antibody levels) to Bm86 after immunisation with a single-dose. The immune responses of the sheep immunised with two lead formulations were significantly higher than sheep immunised with two doses of a TickGard^{PLUS}-like (TGL) formulation after

one year. While sheep are not the natural host of the cattle tick they are a cost effective model for measuring anti-Bm86 antibody responses (De Rose et al., 1999).

The purpose of the current project was to evaluate the capacity of the lead biopolymer-Bm86 formulation from the sheep studies to elicit strong and sustained immune responses in cattle. The immunised cattle would also be challenged with cattle tick larvae once the responses peak to demonstrate that the elicited immune responses can protect these animals from infestation. The second part of the project would determine how long cattle are protected from cattle tick infestation. After immunisation, the immune responses would be monitored at regular intervals, and when the responses begin to decline the cattle would be challenged with cattle tick larvae to demonstrate the time period for which cattle are protected from infestation. The profile of the immune responses of cattle in the first trial would be used to inform the timing of the infestation for the second trial. The timing of the infestation of the cattle in the second trial was expected to be at least six months post-immunisation.

This project addressed the MLA RD&A priority - **reducing the economic impact of cattle tick; define production benefits from effective tick treatment strategies**. The availability of an effective single-dose cattle tick vaccine would also reduce the risk of chemical contamination of beef products resulting from chemical anti-tick treatments. Currently the withholding periods and export slaughter intervals for these treatments are 42 days, with additional time for calves from treated cows. A cattle tick vaccine used as part of an integrated cattle tick management system would enable producers to more effectively get animals to market by reducing the risk of chemical residues. Perhaps more importantly the availability of an effective cattle tick vaccine that provides sustainable long-term control of infestations could permit producers to reduce the Brahman content of their cattle and this would have flow on effects of improved meat quality and easier compliance with MSA programs thus providing the opportunity to increase production value. In a study of dairy cattle, (Jonsson et al., 2000) demonstrated significant differences in liveweight gain of TickGard^{PLUS} vaccinated cattle (52.5 kg) compared to unvaccinated cattle (33.9 kg) during the course of a six-month period of natural cattle tick exposure. Prior to this, Holroyd et al. (1988) reported that Droughtmaster cattle had higher conception rates and weaned heavier calves when cattle ticks were effectively controlled. As Droughtmasters have a high *Bos indicus* genetic content they would be expected have strong natural resilience to cattle ticks, thus demonstrated boosting of production through pest control suggests a vaccine could be equally effective. As a vaccine could provide longer term protection compared to chemical treatments additional benefits might be achieved. While similar studies have not been conducted in beef cattle it does demonstrate the potential benefits of an effective cattle tick vaccine.

The project outcomes strongly align to the Meat Industry Strategic Plan (MISP 2020) priority to improve the ***welfare of animals in our care*** by addressing the imperative to **minimise the impact of endemic disease**. This target aims to reduce the negative impacts of pests and diseases by $50 million and $250 million by 2020 and 2030 respectively through the use of various strategies including vaccines. Improving control of cattle tick could provide a $61 million returned to the cattle industry (Lane et al., 2015). An effective cattle tick vaccine is one way to sustainably achieve this return.

The project outcomes also contribute to the Beef Industry Strategic Plan (BISP 2020) imperative to **minimise the impact of endemic disease** by accelerating the application of **proven industry practice (R&D) for on-farm disease management to contribute to year-on-year reductions in the cost of endemic disease** control. Vaccines are a well proven approach to controlling endemic diseases and are routinely used by the cattle industry to control a range of endemic diseases. Moreover, as discussed previously a cattle tick vaccine was successfully developed and adopted in Australia despite having a sub-optimal vaccination protocol which the current project aimed to address. A single-dose cattle tick vaccine will provide the Australian beef industry with the capacity to reduce losses associated with cattle tick infestations.

This study had two principal aims. The first aim was to determine the capacity of the lead biopolymer formulation to elicit immune responses in cattle and monitor how long these responses persist. The second aim

of the study was to determine if the immune responses elicited by the lead single-dose biopolymer has the capacity to protect cattle from experimental infestation with cattle tick larvae.

2 Project objectives

By 1 November 2019:

Determine the capacity of prototype single dose cattle tick vaccine formulations to induce immune responses in cattle.

Determine the capacity of the lead single dose cattle tick vaccine to protect cattle from experimental infestations at peak immunity.

Determine the capacity of the lead single dose cattle tick vaccine to protect cattle from experimental infestations at six months post-vaccination.

3 Methodology

3.1 Animal Ethics Approval

All experiments described in this report involving cattle were approved by The University of Queensland Production and Companion Animal Ethics Committee, under approval number QAAFI_511_17.

3.2 In Vivo Approval

The use of the biologicals described in this report for in vivo use (in cattle) were approved by the Department of Agriculture as described in Approval Number: 2018/061 [Amendment of 2018/011].

3.3 Cattle immunisation trial 1 – Dose titration

Trial design: Cattle (n = 40, *Bos taurus*, black Angus) were assigned in equal numbers to four treatment groups, blocking for weight. The rectal temperatures for all animals were recorded on Day -2 and Day -1. On Day 0, rectal temperatures were recorded, and 10 mL of blood was collected from all animals. Vaccine formulations were administered to each group as detailed in Table 2.1. A timeline of Trial 1 is provided in Appendix A (Table A9.1)..

Table 2.1 Details of the vaccinations given to trial cattle in experiment 1. Cattle were vaccinated subcutaneously (SC) or intramuscularly (IM).

Group	Vaccine	Number	Route	Bm86 per dose (μg)	Dose 1	Dose 2
1	NPV	10	SC	100	Day 0	Nil
2	NPV	10	SC	200	Day 0	Nil
3	NPV	10	SC	300	Day 0	Nil
4	Conventional	10	IM	50	Day 0	Day 28

Animals were monitored weekly for general health and the injection site was examined for lesion formation in immunised animals. Where at lesion/lump was identified, it was inspected at regular intervals and assessed using the criteria below:

- Visible lesion: Yes / No
- Lesion palpable: Yes / No
- Appearance/description:
- Abscess: Yes / No
- Measure: length, height, width: calculate volume assuming elliptical shape.

On Day 28, blood was collected from all animals (10 mL via jugular vein). A second dose was administered to the Group 4 TGL_50 animals 2 mL subcutaneously (SC) in upper neck region. The Group 4 animals were monitored as previously described. Cattle were subsequently bled every 28 days for the remainder of the experiment. On Day 168 selected animals from each group with low antibody levels were removed from the experiment and euthanised.

On Day 365 the remaining animals were bled and all animals except six from the NPV_300 group were euthanised. To ensure the booster injection sites could be distinguished from these existing lumps, the booster immunisations were given on the right-hand side (no identifiable lumps present prior to Day 365).

At the time the booster dose was administered it was noted that most of the animals had pre-existing lumps of various sizes on the left-hand side of the neck. The six remaining animals from Group 3 were administered a subcutaneous booster dose of the novel polymer to the right-hand side of the neck. The animals were monitored by palpation and measuring. Cattle were subsequently bled every 28 days for the remainder of the experiment. The experiment was terminated at Day 560 with the remaining animals being euthanised. Tissue was collected from the injection sites where the booster injection was administered and submitted for pathological assessment.

3.4 Cattle immunisation trial 2 – Vaccine efficacy

Trial design: Cattle (n = 72, *Bos taurus*, black Angus) were assigned in equal numbers to three groups, blocking for weight. The rectal temperatures for all animals were recorded on Day -2 and Day -1. On Day 0, rectal temperatures were recorded, and 10 mL of blood was collected from all animals. Vaccine formulations were administered to each group as described in Table 2.2. A timeline of Trial 2 is provided in Appendix A (Table A9.2).

Table 2.2 Details of the vaccinations given to trial cattle in experiment 2. Cattle were vaccinated once the novel polymer vaccine (NPV) subcutaneously (SC) or twice with the conventional Tick-guard like (TGL) intramuscularly (IM). All doses were administered in the neck region.

Group	Vaccine	Number	Route	Bm86 per dose (μg)	Dose 1	Dose 2
1	NPV	24	SC	300	Day 0	Nil
2	TGL	24	IM	50	Day 0	Day 28
3	Nil	24	-	-	-	-

All cattle were bled on Day 28 and Day 47. On Day 49, Group 1 animals (n =16) were randomly selected (Animal 3318 was excluded as it did not appear to respond to immunisation) and Group 3 (n =8) were moved into individual pens located within the QC1 animal facility for a seven day acclimatisation period. On Day 56, all cattle in the facility were infested with 4000 cattle tick larvae. This process was repeated on Day 58, Day 61, Day 63, Day 65, Day 68, Day 70, Day 71, Day 72, and Day 75.

From Day 77 to Day 97, engorged adult female cattle ticks dropping from the trial cattle were collected, washed and counted. After transport to the laboratory, the recovered ticks were counted, weighed and assessed for damage typically associated with ingestion of anti-Bm86 antibodies. Data were recorded on an individual animal basis. A selection of ticks (up to 50) from each animal were selected for further analyses, including survival to ovi-position, mass of eggs laid, and hatchability of eggs laid.

Animals not included in the infestation study were bled on Day 85. On Day 98 all experiment 2 animals were bled, euthanised and disposed of via deep burial.

3.5 Detection of anti-Bm86 antibody responses

After collection, the blood samples were allowed to clot at room temperature, the sera harvested and stored at -20 ^{o}C until required. The levels of Bm86 antibody in each serum sample were determined using a standard ELISA assay. All serum samples were diluted 1:800 for screening purposes. A control serum from a previous trial was used as a reference serum sample (diluted 1:800) to facilitate comparisons between ELISA plates and the reactivity of samples collected at each sampling time-point.

3.6 Testing of emulsion and Bm86 antigen formulation stability

3.6.1 Stability of the microhydrogel formulation

The Biopolymer-Microhydrogels in Montanide ISA61VG formulations (novel vaccine formula, NPV) were produced according to Al Kobaisi and Mainwaring (2019) together with the Bm86 antigen. Confocal laser scanning microscopy (CLSM) was used in the previously used to verify the formulation microstructure and Bm86 distribution as a partition between free and micro-encapsulated Bm86. As a quality control (QC) test for these formulations, this new technique provided verification of both the hydrogel emulsion dispersion and the partition of Bm86 antigen immediately available upon injection as a priming dose compared to that progressively available for persistent delivery. To address the issues of longer-term formulation stability under imposed environmental and handling conditions.

The longer term stability of the single injection formulation was evaluated with Dynamic Laser Scattering (DLS) of freshly prepared samples of the microhydrogel in emulsion formulations together with samples stored for 5 months at 20 °C, and when the Bm86 antigen loadings were 100, 200, and 300 µg per 3.5 mL dose. Samples were diluted and re-suspended in the adjuvant Montanide ISA61VG addition. A HORIBA LA-960 instrument, operating via laser diffraction was used to evaluate the size distribution of each of these samples. This method required the refractive index of both the dispersant and dispersion phases for which literature values of 1.460 and 1.336 for Montanide ISA and chitosan were used respectively.

3.6.2 Qualitative ELISA evaluation of the formulated Bm86 stability

For Bm86 extraction from the formulations, fully formulated microhydrogel emulsions were de-emulsified with benzyl alcohol according to Miles and Saul (2005). Benzyl alcohol was added to the initial formulation to provide a 1:9 volumetric ratio, followed by vortexing for 10-20 min. The solution was centrifuged at 14,000*g* for 10 min. Free Bm86 was recovered from the middle aqueous layer, while the hydrogel containing the bound Bm86 fraction formed the bottom (pellet) layer (Fig. 3.1).

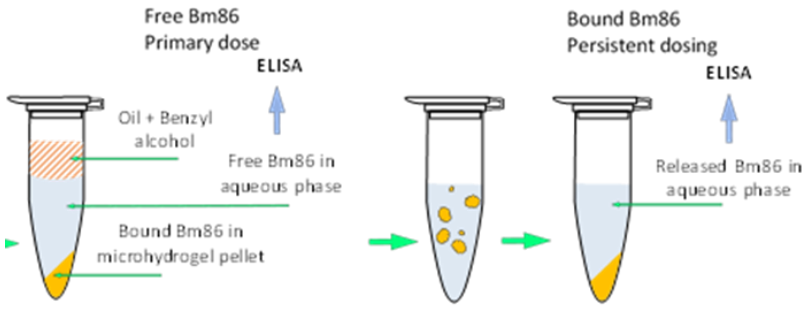


Fig. 3.1: *Graphical representation of the Bm86 extraction protocol from the microhydrogel in emulsion formulation.*

The recovered Bm86 was analysed using ELISA assays to evaluate any loss of antigenicity over time using monoclonal antibodies. The recovered Bm86 from the different phases of the extraction process (Fig. 3.1) was used on standard ELISA plates (Nunc MaxiSorp™, ThermoFisher Scientific) at 100 µL of Bm86 antigen (1 ng/µL) in phosphate buffered saline (PBS) overnight at room temperature. The Bm86 from the formulations was evaluated against a number of standards containing potential interferences. These were: (a) blank bulk hydrogel syneresis liquid (containing traces of soluble biopolymer hydrogel), (b) Bm86 extracted from microhydrogel in emulsion in the presence of the aqueous syneresis liquid which formed during formulation, and which had been in contact with the Montanide ISA61VG adjuvant oil. Two monoclonal antibodies (RC2 and RD4), were used to detect recovered native and denatured Bm86 respectively.

4 Results

4.1 Duration of antibody responses (Trial 1)

4.1.1 Safety primary immunisation

No site reactions were observed in any of the cattle immunised with the NPV or the CV doses between Day 0 and Day 365.

4.1.2 Antibody responses from Day 0 to Day 365 (Single dose)

The antibody responses for the cattle in Trial 1 were monitored from Day 0 to Day 365 (Fig. 4.1 to Fig. 4.4).

Overall immune responses within the groups immunised with the NPV were highly variable. In the early phase of the trial there appeared to be minimal differences between any of the groups (Fig. 4.1 to Fig. 4.4). From Day 215 the mean antibody levels in the NPV with 200μg of Bm86 (NPV_200) and NPV with 300μg of Bm86 (NPV_300) groups were higher than those detected in the NPV with 100μg of Bm86 (NPV_100) (compare Fig. 4.1a, 4.2a and 4.3a). Interestingly, from Day 301 onwards the means of the NPV groups were reflective of the amount of Bm86 antigen in the formulations, with the NPV_300 and the NPV_100 having the highest and lowest means respectively (Fig. 4.1 to Fig 4.3). In comparison to the TGL group, the means of the NPV groups were higher across the majority of the trial from Day 140 onwards (Fig. 4.4). Despite these trends in the data, no significant differences were detected between the any of the groups.

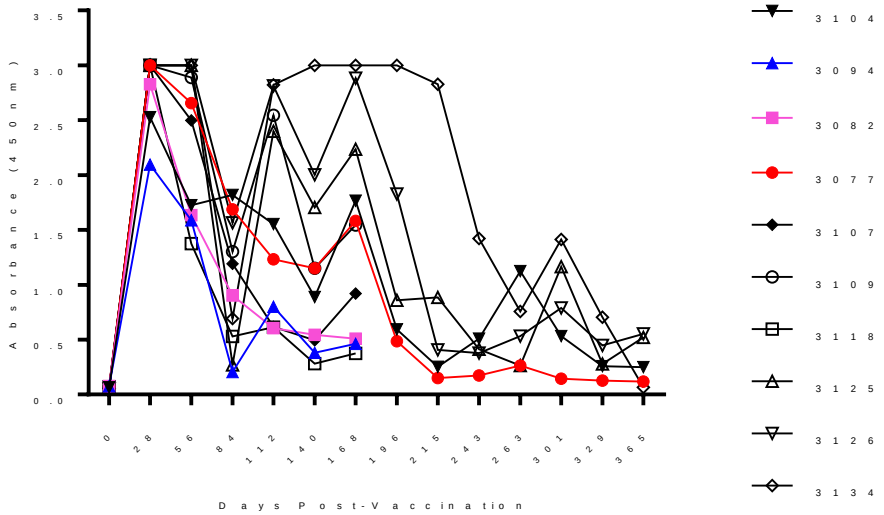
The antibody responses for each animal in the treatment groups are shown on separate graphs for clarity (Fig. 4.1 to Fig 4.4). Comparison of these antibody responses reiterates the similarity of the profiles across the duration of the sampling period. There is evidence of a multi-phasic release of the Bm86 antigen, with relative antibody peaks at Day 196, Day 243 and Day 301 for the NPV_300 group (Fig. 4.3). While similar peaks are evident for the NPV_100 and NPV_200 groups they are not as prominent (Fig. 4.1 & 4.2). These results are consistent with the proposed mechanism of action the NPV, with periodic release of antigen/adjuvant complexes overtime as a result of diffusion from the polymer complex and/or degradation of the polymer matrix, resulting in restimulation of the immune response.

Considerable variability between animals within the various treatment groups was evident from the group plots. This is was further demonstrated when the antibody levels for individual animals are plotted for each group (Fig. 4.1A to Fig 4.4A). At the animal level, these plots suggested all animals initially responded well to the NPV formulations by Day 28. However, the responses for two to four animals from each group the response was short-lived, with the antibody levels rapidly decaying from Day 28 to Day 84. While not statistically significant, the antibody responses of the animals in the NPV_200 (Fig. 4.2A) and NPV_300 (Fig. 4.4A) were maintained at higher levels for the duration of the experiment.

At the end of the trial (Day 365), four of the remaining six animals in the NPV_300 had relative antibody levels near or above 0.8 absorbance units (Fig. 4.4A), whereas only one of the five remaining animals immunised with NPG_200 was clearly above 0.8 absorbance (Fig. 4.2A). As expected, the antibody

responses for the TGL group peaked at Day 56 (except for two animals), followed by a steady rate of decay until Day 365 (Fig. 4.4A and B). After Day 215, the remaining animals in this group had antibody levels well below the 0.8 absorbance units (Fig. 4.4A).

A)



B)

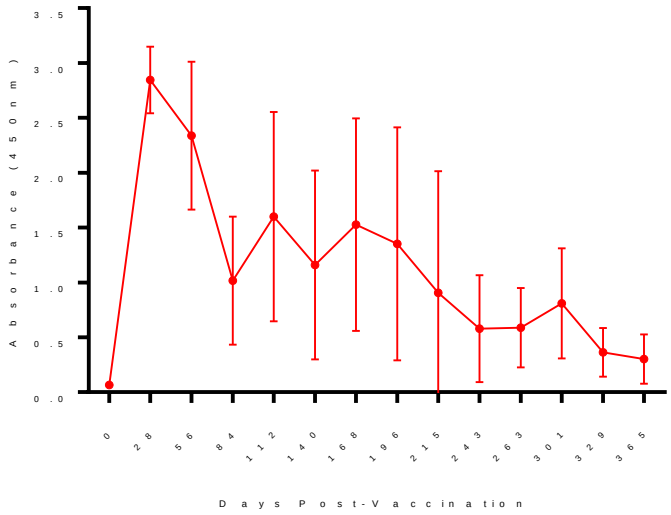
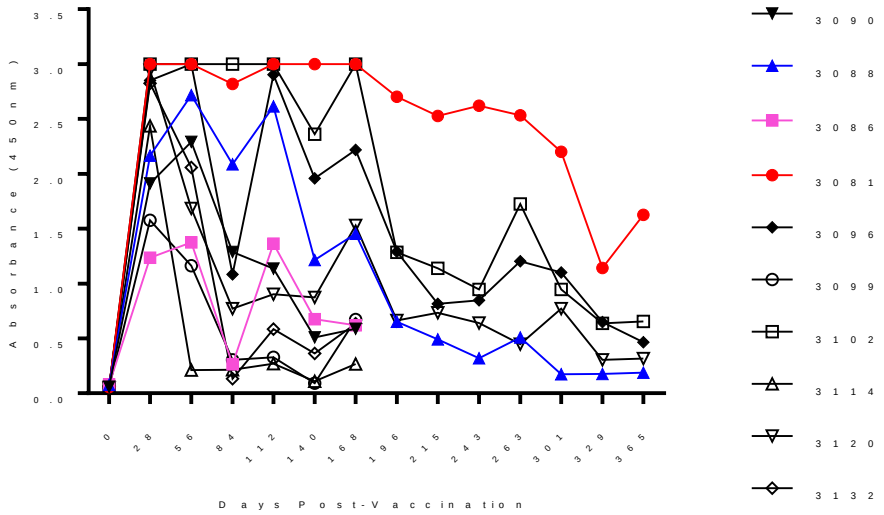


Fig. 4.1 Antibody responses in cattle (n=10) immunised with a single dose of the novel polymer formulation with 100 μg of Bm86. A) Individual animal optical density data for each time point. B) Mean optical density for the group at each time point. Error bars represent one standard deviation. Four animals were culled this group on Day 168.

A)



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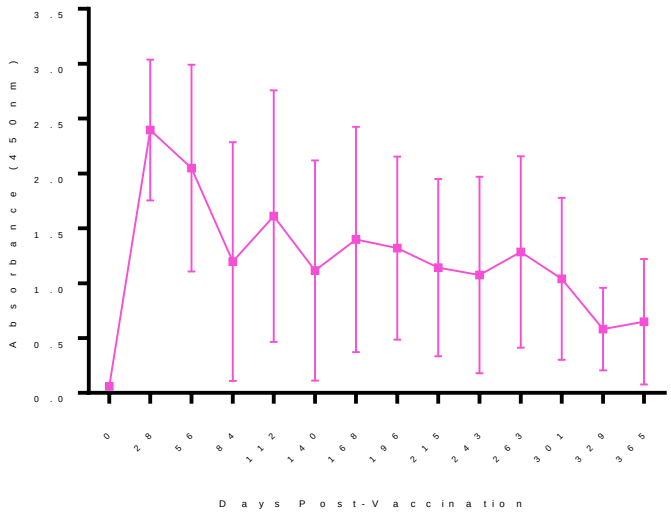
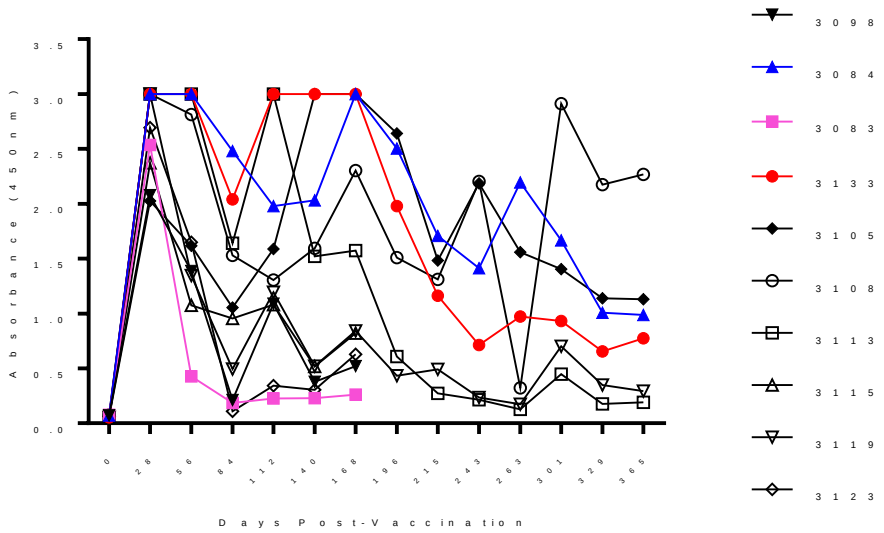


Fig. 4.2 Antibody responses in cattle (n=10) immunised with a single dose of the novel polymer formulation with 200 μg of Bm86. A) Individual animal optical density data for each time point. B) Mean optical density for the group at each time point. Error bars represent one standard deviation. Five animals were culled from this group on Day 168.

A)



B)

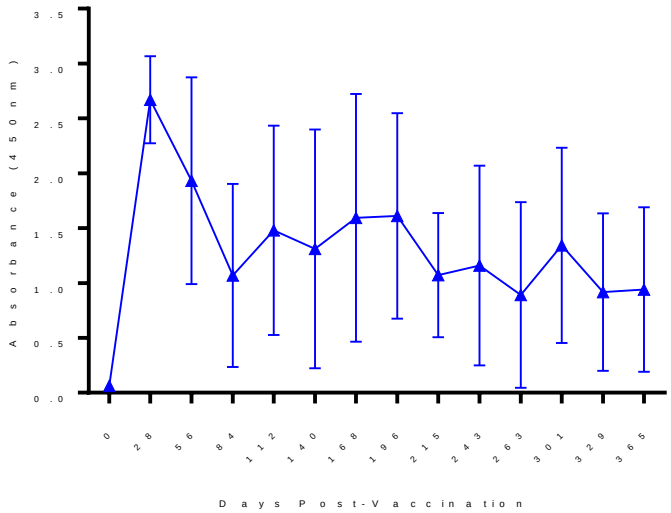
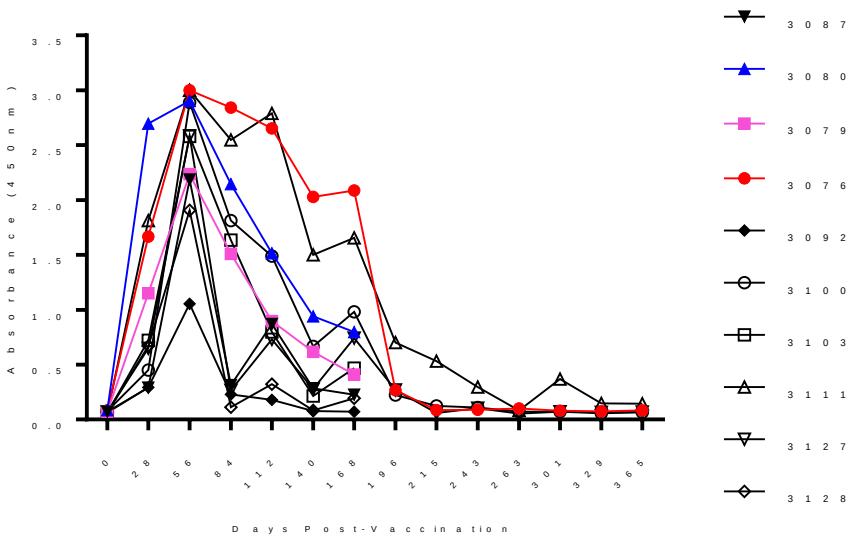


Fig. 4.3 Antibody responses in cattle immunised with a single dose of the novel polymer formulation with 300 μg of Bm86. A) Individual animal optical density data for each time point. B) Mean optical density for the group at each time point. Error bars represent one standard deviation. Four animals were culled from this group on Day 168.

A)



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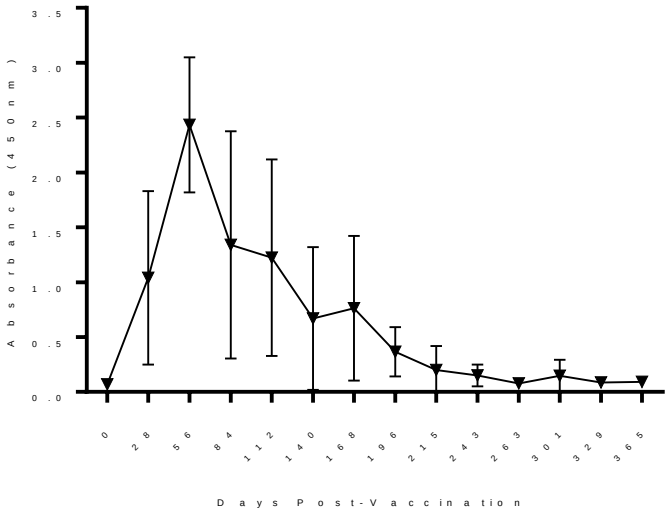
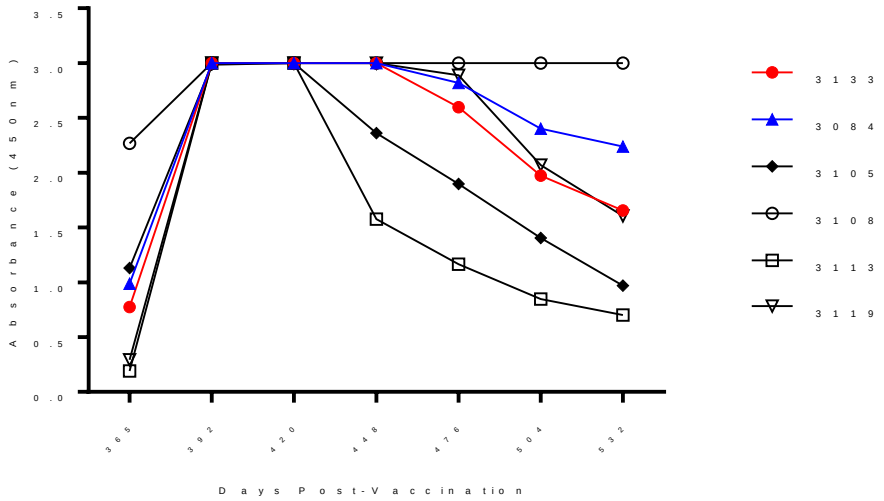


Fig. 4.4 Antibody responses in cattle immunised with the conventional double dose tick guard like formulation with 50 µg of Bm86 in each dose. Doses were administered on Day 0 and Day 28. A) Individual animal optical density data for each time point. B) Mean optical density for the group at each time point. Error bars represent one standard deviation animals were culled from this group on Day 168.

4.1.3 Antibody responses following booster immunisations (Day 365 to Day 532)

The antibody responses of the cattle receiving the booster injection on Day 365 are shown in Figure 4.5A and 4.5B. All of the animals responded with very strong antibody levels for at least 56 days (Day 420) following the booster immunisation (Fig. 4.5A). The antibody responses of Animal 3108 did not decrease in the six months following the booster immunisation (Fig. 4.5A). This animal had the highest levels of antibody at Day 365 prior to the booster immunisation. Animal 3113 had the lowest antibody levels prior to the booster immunisation and it also had the lowest levels of antibody at the end of the experiment (Fig. 4.5A).

A)



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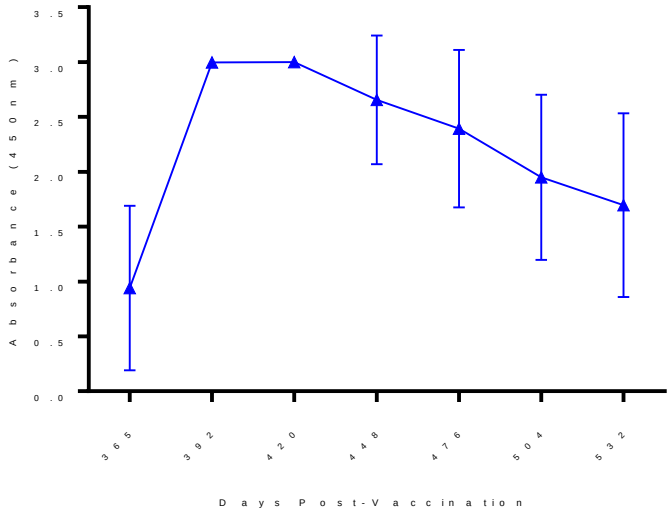


Fig. 4.5 Antibody responses in cattle (n=6) immunised with a booster dose of the novel polymer formulation with 300 μg of Bm86 from Day 365 to Day 532. A) Antibody responses of individual animals. Animal Identification numbers shown. B) Average antibody responses for all animals. Error bars represent one standard deviation.

4.1.4 Safety booster immunisation

Approximately two hours post-injection, swelling was observed at the injection site on several animals after administration of the booster dose. Observations of the cattle suggest they were not experiencing any discomfort from the swelling (e.g. not attempting to rub the area). Similarly behavioural observations on the days after the booster injection failed to detect any changes in eating/drinking or demeanour of the animals.

The properties of these lumps are summarised in Table 4.1. The elliptical volumes of the lumps were highly variable, on Day 369 (four days post-boost) they ranged from 875 cm^3 to 1 cm^3 (Table 4.1). The volume of the lumps appeared to be largest at Day 372 for most animals. Towards the end of this phase of the experiment the lumps in all animals appeared to be reducing in size. This was particularly evident for Animal 3119, where no injection site lump was detected on either Day 504 or Day 532. While the lumps were very pronounced in the majority of the animals, only one ruptured (Animal 3084, Day 272). Similarly, as the behaviour of the animals did not change and lumps were stable, no interventions were undertaken, such as draining, as this could have created open wounds. Given the cattle were housed in open pens, open wounds may have increased the risk of secondary infections leading to complications.

Based on the calculated elliptical volumes, the lumps appeared to increase in size at the end of the trial (Table 4.1). These observations were inconsistent with the reports of the animal care staff for the previous inspection times that the lumps were visually stable. This discrepancy is attributed to being able to more accurately measure the lumps on Day 532 during post-mortem examinations. Whereas other measurements at the other time points were taken on live animals within the constraints of a crush.

The results of the pathologist's assessment of the lump biopsies are shown in Table 4.2. The overall pathological findings suggest the reactions were consistent with hypersensitivity and/or allergic reactions. While the pathologist suggested these reactions may be attributable to the adjuvant in the immunisation formulations, the pathologist was not aware of the compositions of the formulations, only that the tissues were taken from the injection sites from a vaccine study.

Photographs of the injection site lumps at the end of the trial (Day 532) are shown in Fig. 4.6. These photographs illustrated the highly variable nature of these lumps. The most pronounced lump was observed on Animal 3113 (Fig. 4.6D). In comparison no lump was identified in the injection site area for Animal 3119 (Fig. 4.6E). The level of site reaction to the booster immunisation (lesion sizes in Table 4.1) did not appear to correlate with the detected antibody responses on day 352 shown in Fig. 4.5A.

Table 4.1 Properties of the injection site lumps in cattle following administration of a booster injection with the NPV_300 at Day 365.

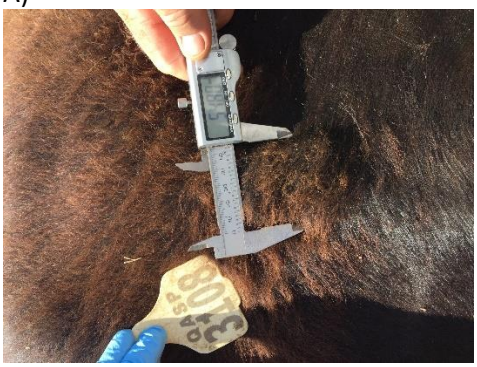
		Animal ID					
Day*	Properties	3108	3084	3105	3113	3119	3133
369	Texture	Firm	Hard	Hard	Firm	Hard	Hard
	Ruptured	No	No	No	No	No	No
	Volume (cm^3)	348	603	814	875	1	656
372	Texture	Hard	Not done	Soft	Hard	Firm	Firm
	Ruptured	No	Yes	No	No	No	No
	Volume (cm^3)	294	3,670	919	852	33	852
504	Texture	Small	Firm	Firm	Firm	Absent	Firm
	Ruptured	No	No	No	No	No	No
	Volume (cm^3)	42	26	105	105	0	26
532†	Texture	Firm	Firm	Firm	Firm	Nil	Firm
	Ruptured	No	No	No	No	No	No
	Volume (cm^3)	249	252	366	1,986	0	239

*Observations were done on Day 375 and Day 378, with no visual changes compared to Day 372. Lumps were not measured at these time points. †Injection site lumps measured post-mortem.

Table 4.2 Summary of the pathological observations made on tissues recovered from the injection site lumps of trial animals at the end of the trial (Day 532)

Tissue	Animal ID					
Assessment	3108	3084	3105	3113	3119	3133
Appearance	firm and white effacing blood vessels	firm and white accumulation of	firm and white pyogranulomas with	firm white and fibrous blood vessel with	No lump	Firm and white no margins, nodular
Histology	are granulomas rimmed by eosinophils, vessel walls expanded and replaced by macrophages, lymphocytes, oedema.	macrophages admixed with lymphocytes, vasculitis, mineralisation, granuloma/abscess wall	central area of viable and degenerate neutrophils, eosinophils and macrophages.	distorted wall, tunica intima and media up to 4 times normal thickness and filled with eosinophils, macrophages and lymphocytes, oedema, spindle cells, fibrosis	Not applicable	aggregates with high numbers of lymphocytes and some macrophages with eosinophils and moderate number of plasma cells
Morphologic diagnosis	Chronic multifocal eosinophilic granulomatous vasculitis and lymphoplasmacytic dermatitis	chronic multifocal perivascular lymphocytic, vasculitis and granulomatous dermatitis, pseudocyst	Chronic multifocal to coalescing pyogranulomas with intralesional eosinophils, marked to severe	Chronic multifocal perivascular lymphocytic and eosinophilic dermatitis/vasculitis	Not applicable	Chronic multifocal perivascular lymphocytic and eosinophilic dermatitis/panniculitis
Comments	Histopathological findings indicated a chronic inflammatory process that was often centred around and affecting blood vessels. Eosinophils and macrophages were the predominating infiltrate on all animals with exception of animal #3133, where an eosinophilic and lymphoplasmacytic infiltrate was most present, however, mild to moderate. In absence of intralesional foreign material or parasites and given history of vaccine-site associated lesions, the vasocentric distribution of the eosinophilic granulomatous inflammation, in special on animals #3133, 3113, 3105 and 3084, were suggestive of mixed type I and type IV hypersensitivity. Nonetheless, pyogranulomas with intralesional eosinophils noted on animal #3105, were most compatible with chronic reaction to allergenic foreign material, possibly adjuvant but other possibly non-vaccine related material or cause cannot be completely excluded.					

A)



B)

C)

D)

E)

F)

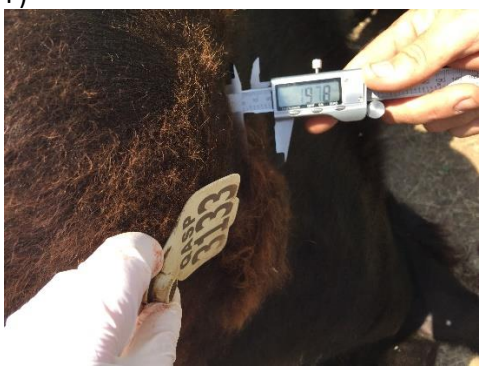
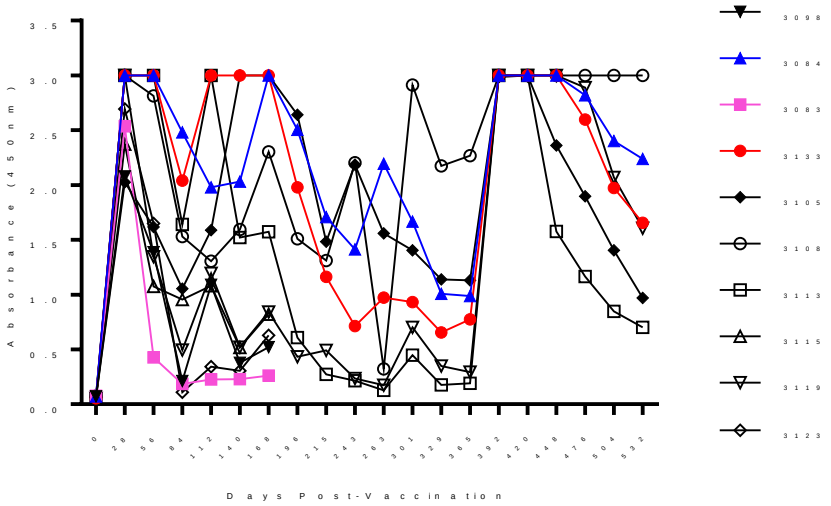


Fig. 4.6 Photographs of the booster immunisation injection site lumps in study cattle at the end of the trial (Day 532). Panel A: Animal 3108; Panel B: Animal 3084; Panel C: Animal 3105; Panel D: Animal 3113; Panel E: Animal 3119; Panel F: Animal 3133.

4.1.5 NPV_300 combined antibody responses: Day 0 to Day 532

Fig. 4.7 shows the combined immune responses from the NPV_300 group across the duration of the study.

A



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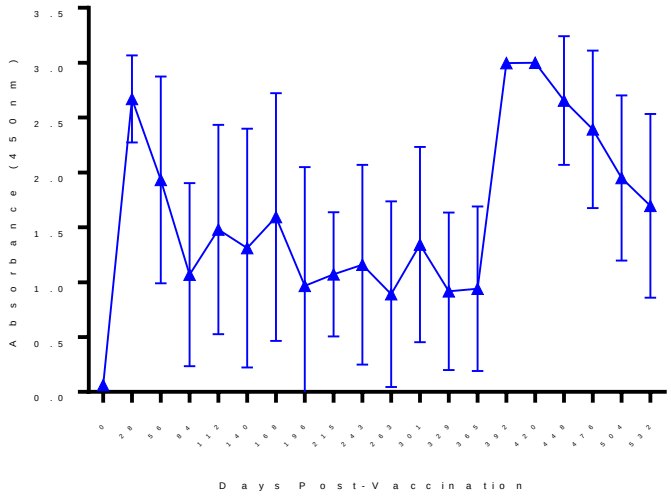


Fig. 4.7 The antibody responses in cattle (n=10) immunised with a single dose of the polymer formulation with 300 μg of Bm86 from Day 0 to Day 532. A) Individual animal optical density data for each time point. B) Mean optical density for the group at each time point. Error bars represent one standard deviation. Four animals were culled from this group on Day 168. Booster immunisations were administered to the remaining six animals on Day 365.

4.2 Protection from infestation (Trial 2)

4.2.1 Antibody responses

The antibody responses for the Trial 2 animals are shown in Fig. 4.8.

As with the previously reported Trial 1, higher levels of Bm86 specific antibody were detected at Day 28 post-immunisation in the NPV_300 group compared to the TGL_50 group (Fig. 4.8A). Once the TGL_50 group received the second injection the responses increased to levels similar to those in the NPV_300 group. At Day 97 post-immunisation the levels of Bm86 antibodies were similar in the NPV_300 and TGL_50 groups (Fig. 4.8A). No Bm86 specific antibodies were detected in the sera samples from the unimmunised control group during the experiment (data not shown).

Within the NPV_300 immunised group in Trial 1, there had been considerable variation between the individual animals (Fig. 4.3A). Similarly, in Trial 2, a broad range of responses to Bm86 were also detected (Fig 4.8B and 4.8C). The 16 animals were randomly selected from the NPV_300 group for the cattle tick larvae infestation. One animal 3318 was excluded from this selection process, as it did not appear to respond to immunisation (Fig. 4.8C). As expected, no Bm86 specific antibodies were detected in any of the control animals Fig. (4.9).

The differences in the levels of Bm86 specific antibody between the animals (n=16) selected for infestation and the animals (n=8) are shown in Fig. 4.9.

No adverse reactions were recorded for the any of the animals immunised with either the NPV_300 (n=24) or TGL_50 (n=24) formulations in Trial 2.

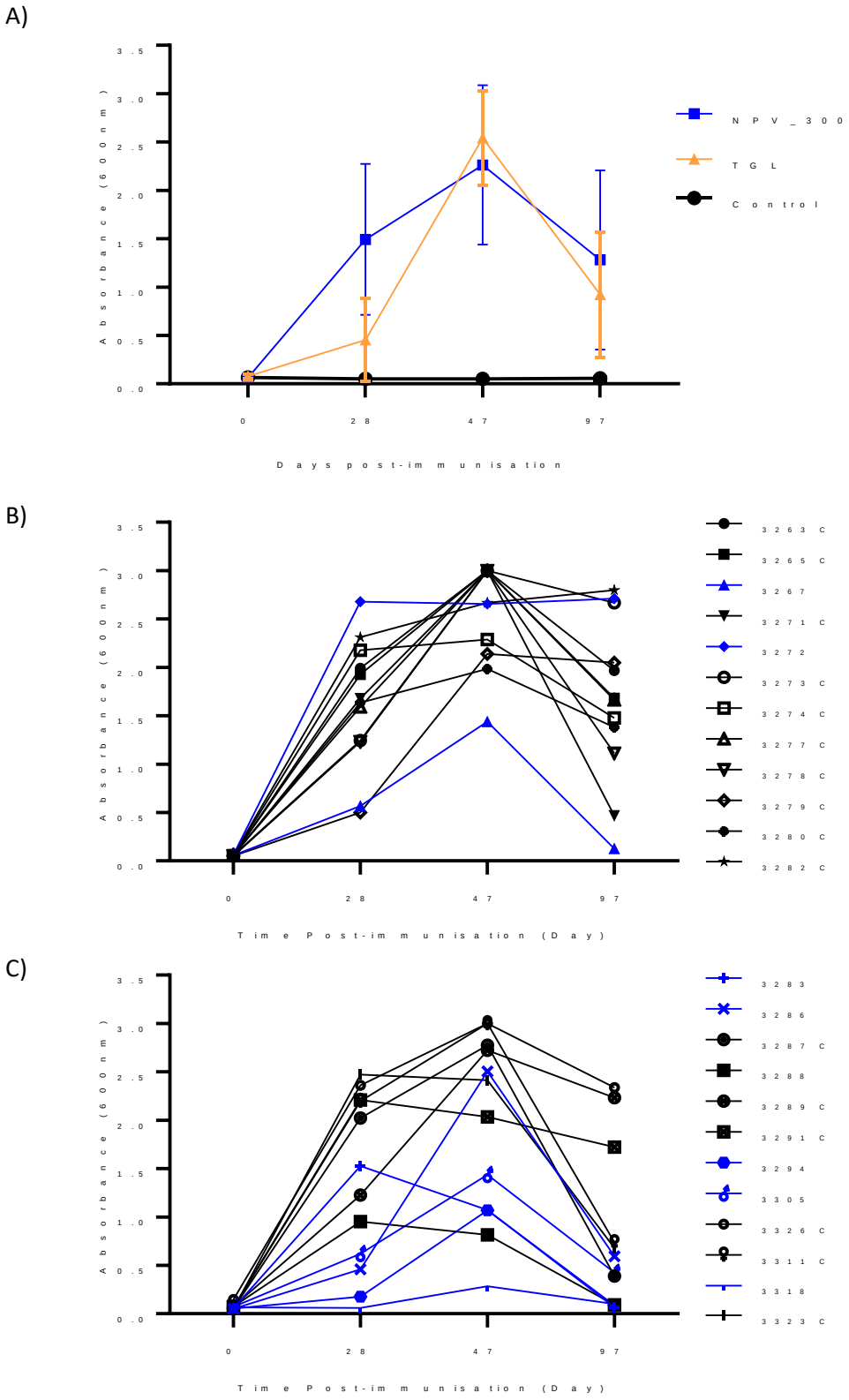
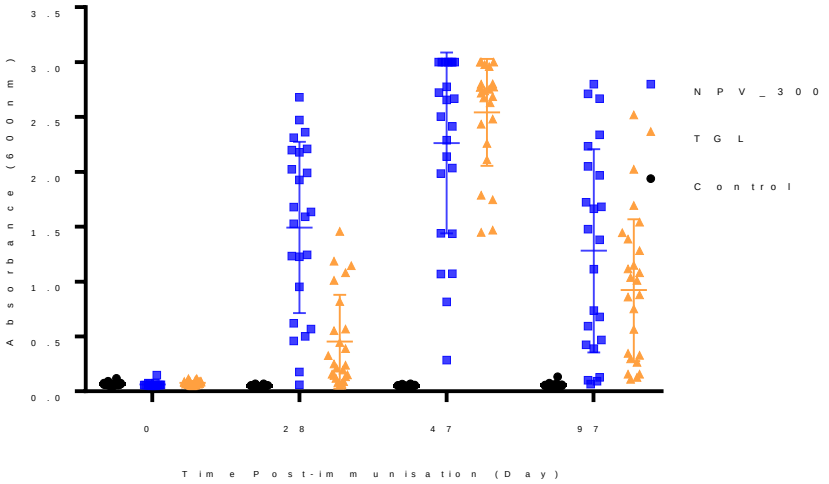


Fig. 4.8: The level of Bm86 specific antibody (IgG) circulating in cattle during Trial 2. Cattle were immunised with either the novel polymer vaccine containing 300 μg of Bm86 (NPV_300) on Day 0 or the tick guard like (TGL) formulation on Day 0 and 28. A) Mean Bm86 specific antibody levels in the sera collected from the trial groups. The average OD600 values are shown for each time-point. Error bars represent one standard deviation. B) & C) The Bm86 specific antibody levels in the serum from

each animal immunised with NPV_300 formulation. Animal identification numbers are shown; “C” denotes challenged animals, blue symbols and lines denote animals not challenged .

A)



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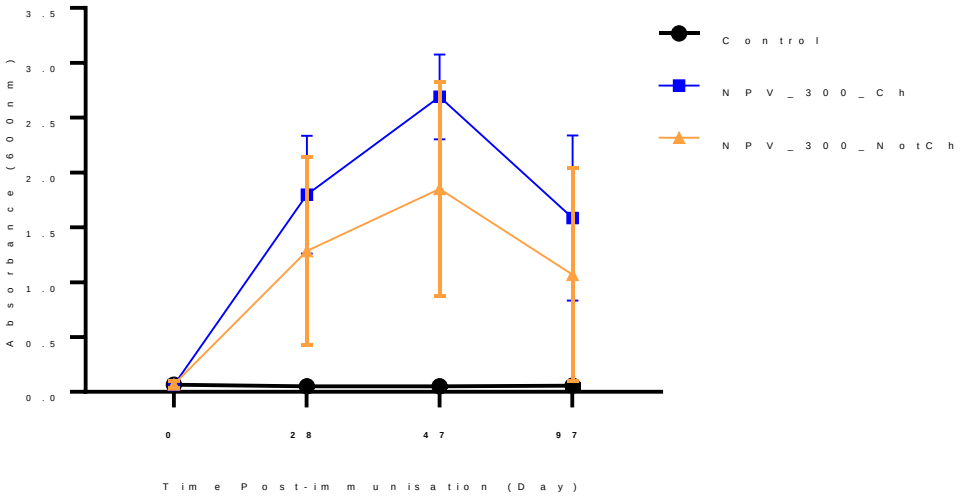

Fig. 4.9: Selection of cattle to be infested with cattle tick larvae. A) Scatter plot of the OD600 vales for all animals in each treatment group in the study. B) Comparison of the antibody levels of cattle either selected for challenge (NPV_300_Ch, n=16) or not selected challenged (NPV _300_NotCh, n=8). The mean OD600 values for are shown for each time-point with one standard deviation.

4.2.2 Infestation with cattle tick larvae

4.2.2.1 Recovery of adult cattle ticks

Few ticks were recovered in the early stages of tick collection from all of the cattle in the study, regardless of treatment group (data not shown). Fig. 4.10 shows the average number of adult ticks collected for the control and NPV_300 groups. While there was a trend for the fewer adult ticks to be recovered from the vaccinated group, these differences were not statistically significant for most collection days. The exception to this was Day 77, when significantly fewer ticks were collected from the immunised group ($p<0.05$). Given the passive nature of Bm86 based immunisation, which reduces the overall fitness of the mature cattle ticks rather than killing them, these results were not surprising.

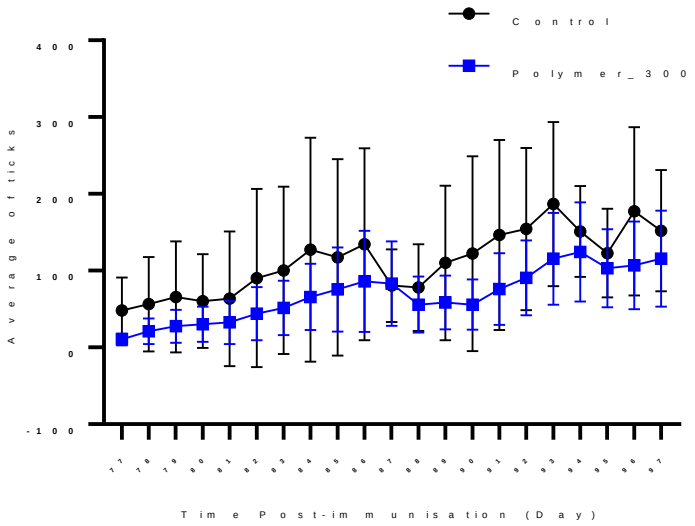

Fig. 4.10: The average number of cattle ticks recovered from the trial cattle between Day 77 and Day 97 post-immunisation. The mean number of adult ticks recovered on each day for each treatment group is shown. Error bars represent one standard deviation of the mean.

4.2.2.2 Mean weights of cattle ticks

The next parameter to be evaluated was the mean cattle tick weights from the control and immunised animals (Fig. 4.11). The cattle ticks recovered from the immunised group were significantly lighter compared to those recovered from the unimmunised group on all collection days ($p<0.001$, all days).

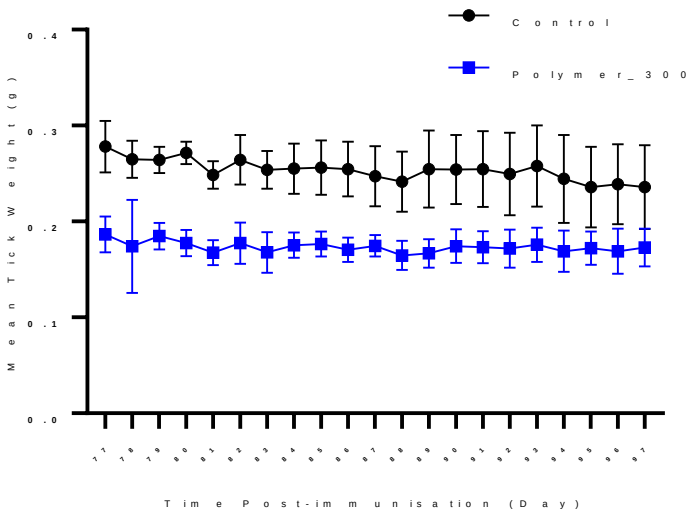

Fig. 4.11: The mean weights of the cattle ticks recovered from the trial cattle between Day 77 and Day 97 post-immunisation. Error bars represent one standard deviation. The mean weight of cattle ticks collected from the immunised group was significantly less on all experimental days ($p<0.001$).

4.2.2.3 Percentage of damaged cattle ticks recovered

A representative sample of cattle ticks recovered from each group on Day 97 are shown in Fig. 4.12. The undamaged cattle ticks from the control group were a green-brown colour in appearance with yellowish stripes (urate crystals), dimpled carapaces and clear/yellow legs (Fig. 4.5A). In contrast the cattle ticks from the immunised group, were red-brown in appearance, little or no yellow stripes, generally smooth carapaces and mostly reddish legs (Fig. 5.5B).

A) B)

Fig. 4.12: Characteristic appearance of cattle ticks recovered during the infestation trial, collect on Day 97. A) Cattle ticks from an unimmunised animal. B) Cattle ticks from an immunised animal.

The average percentage of damaged cattle ticks recovered on each collection day from the control and immunised groups are shown in Fig. 4.13. A significantly higher percentage of damaged cattle ticks were recovered from the immunised group compared to the unimmunised group on all collection days ($p<0.001$ all days, Fig. 4.13). The percentage of damages ticks collected from the immunised cattle appeared to be increasing over time (Fig. 4.13). To further explore this observation a linear regression analysis was performed on the data. The analysis confirmed a positive correlation between the percentage of damaged ticks collected and days post-immunisation (Fig. 4.14). The correlation co-efficient, R^2, was 0.756 with the slope of the linear regression varying significantly from zero ($p<0.0001$).

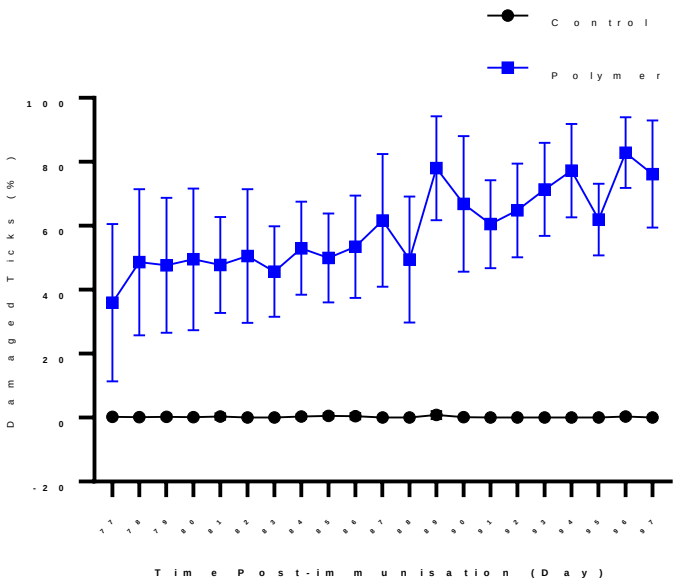

Fig. 4.13: The percentage of damaged cattle ticks recovered from the trial cattle between Day 77 and Day 97 post-immunisation. Error bars represent one standard deviation.

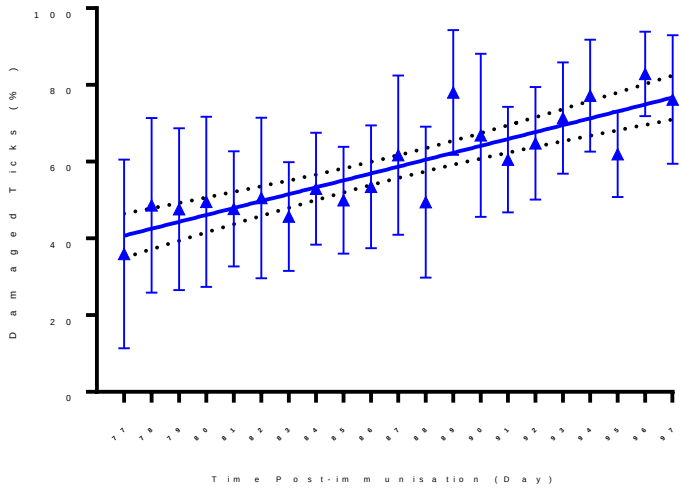

Fig. 4.14: Linear regression analysis of the percentage of damaged cattle ticks recovered from cattle immunised with the polymer formulation as a function of days post-immunisation. The dotted lines illustrate the 95% confidence intervals.

4.2.2.4 Cattle tick survival to oviposition

Significantly ($p<0.01$) fewer cattle ticks survived to the oviposition stage for all collection days (Fig. 4.15).

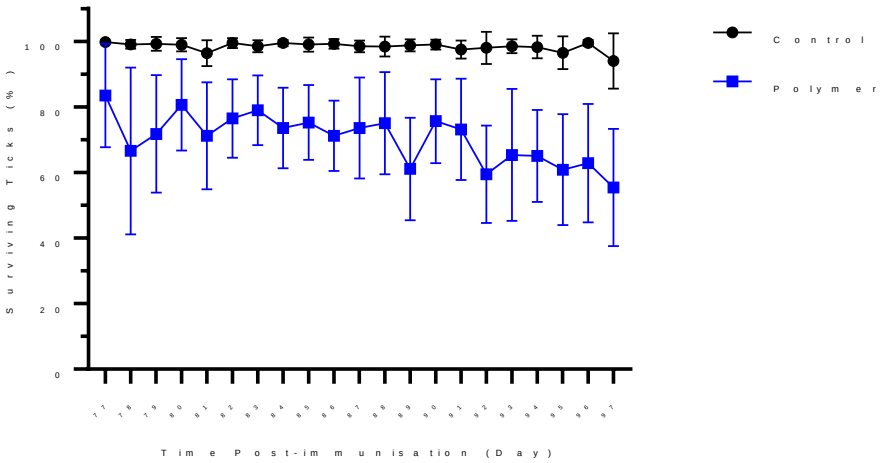

Fig. 4.15: The mean survival percentage of cattle ticks recovered from the trial cattle between Day 77 and Day 97 post-immunisation. The means of surviving cattle ticks differed significantly for each day ($p<0.01$). Error bars represent one standard deviation.

4.2.2.5 Capacity of recovered ticks to lay eggs

Across all collection days the cattle ticks from the immunised group, on average, laid significantly lighter egg masses per gram of incubated cattle ticks (Fig. 4.9). The smallest and largest differences between the between the average mass of eggs laid were 0.0715g ($p<0.05$) and 0.4042g ($p<0.001$) on Day 77 and Day 96, respectively (Fig. 4.16).

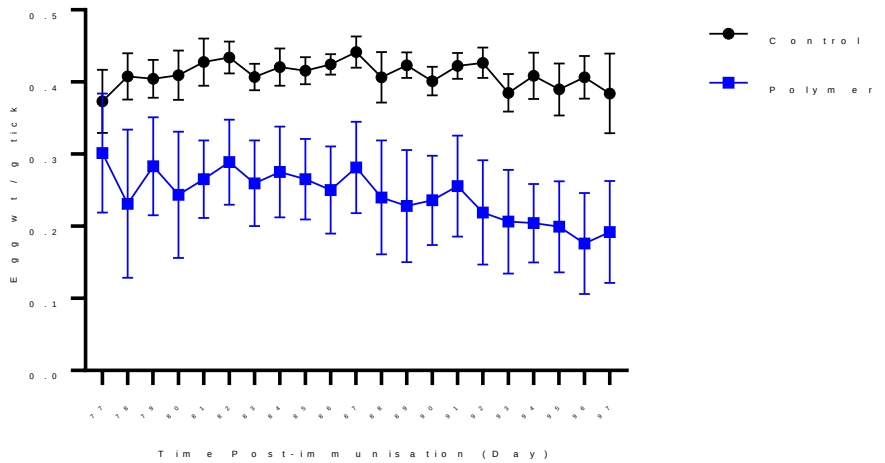

Fig. 4.16: The mean weights of the eggs laid by cattle ticks recovered from the trial cattle between Day 77 and Day 97 post-immunisation. Error bars represent one standard deviation.

4.2.2.6 Viability of cattle tick eggs (Day 7)

The egg viability between the two treatment groups was quite similar, however, significant differences between the average egg viability were detected on Day 83, Day 85, Day 86, Day 88, Day 90 to Day 94 and Day 96 post-immunisation (Fig. 4.17).

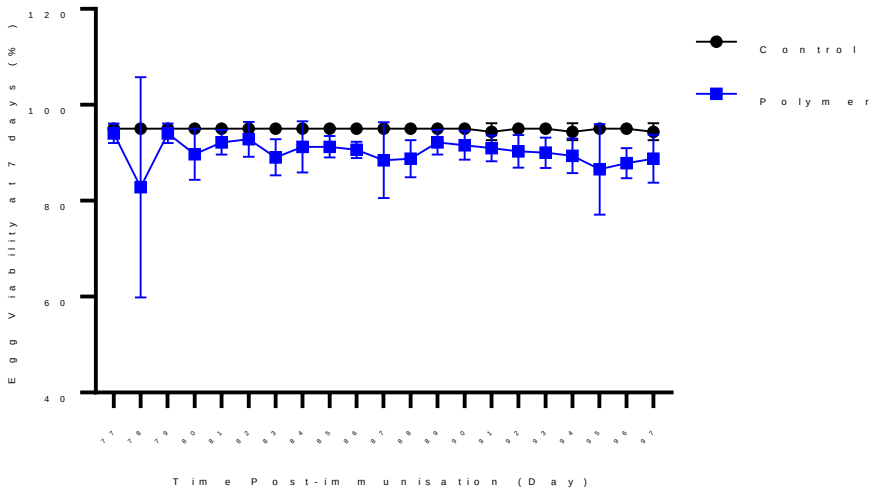

Fig. 4.17: Viability of cattle tick eggs seven days after laying from the trial cattle between Day 77 and Day 97 post-immunisation. The average estimated viability of laid eggs by adult ticks recovered from the unimmunised control cattle (n=8) and the immunised cattle (n=16) are shown. Error bars represent one standard deviation.

4.2.2.7 Viability of cattle tick eggs at hatch (Day 35)

The viability of the eggs laid by the incubated cattle ticks was visually assessed at Day 35 post-lay (Fig. 4.18). The egg viability between the two treatment groups were quite similar, however, significant differences between the average egg viability were detected on Day 81, Day 83, Day 85, Day 93, Day 95 and Day 96 post-immunisation (Fig. 10).

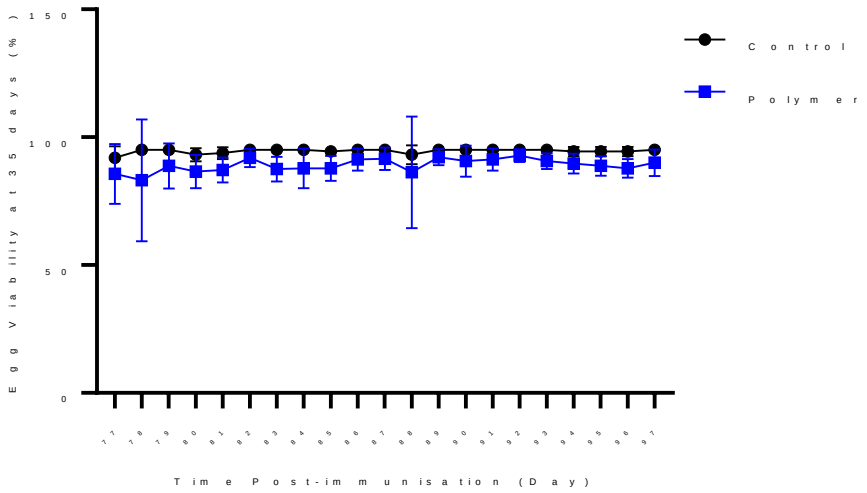

Fig. 4.18: Viability of cattle tick eggs 35 days after laying by cattle ticks collected dropping from cattle between Day 77 and Day 97 post-immunisation. The average estimated viability of laid eggs by adult ticks recovered from the unimmunised control cattle (n=8) and the immunised cattle (n=16) are shown. Error bars represent one standard deviation.

4.2.3 Estimation of vaccine efficacy of the *novel polymer vaccine*

The efficacy of the novel polymer vaccine was estimated using two accepted methodologies. The first was based on the reproductive estimates and the average number of ticks collected. This yielded a cumulative vaccine efficacy of 67.6% from Day 77 to Day 97 post-immunisation (Fig. 4.19). The highest and lowest daily vaccine efficacy were detected on Day 77 (84.4%) and Day 87 (37.2%), respectively.

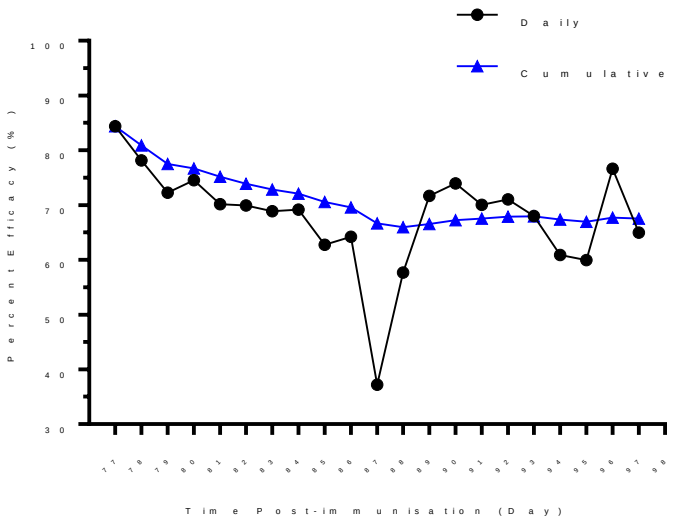

Fig. 4.19. Estimates of vaccine efficacy. The vaccine efficacy was estimated using the reproductive estimates and the average number of ticks collected. The daily efficacy and cumulative efficacy are shown.

The second efficacy estimate was based on the reproductive estimates and the average weight of the ticks recovered from the treatment groups. This yielded a cumulative vaccine efficacy of 76.9% from Day 77 to Day 97 post-immunisation (Fig. 4.19). The highest and lowest daily vaccine efficacy were detected on Day 77 (89.6%) and Day 87 (54%), respectively.

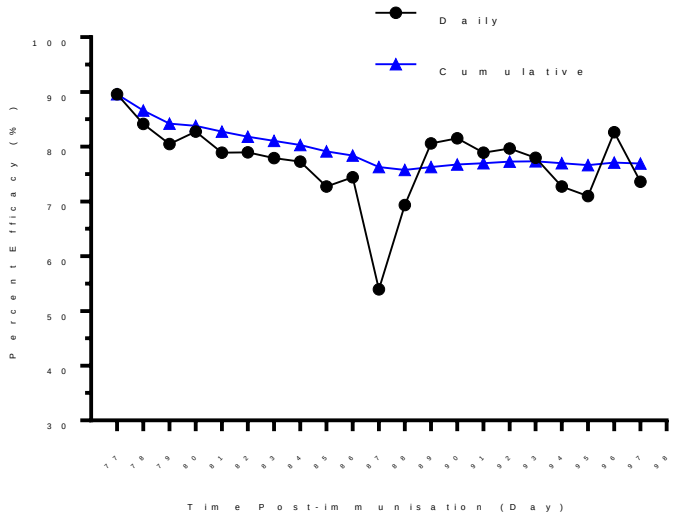

Fig. 4.20. Vaccine efficacy. The vaccine efficacy was estimated using the reproductive estimates and the average weight of ticks collected. The daily efficacy and cumulative efficacy are shown.

4.3 Microhydrogel - quality control under environmental and handling conditions

4.3.1 Stability of the microhydrogel formulation

Emulsion stability of the final microhydrogel formulation containing Bm86 antigen was evaluated over a five-month period when stored at room temperature (20 °C) and showed a very high degree of stability against irreversible flocculation and emulsion breakdown. The mean hydrogel emulsion diameter only changing from about 0.02 to 0.04 microns over this five-month period.

Stability of the Bm86 antigen epitopes within these microhydrogel formulations was evaluated by a modified qualitative ELISA procedure since some interference occurred from both residual traces of biopolymer within the aqueous syneresis phase and the Montanide oil used as an adjuvant and emulsifier. The de-emulsification, extraction and the modified ELISA shows that about 40 - 50% of the Bm86 antigen is present free in the aqueous phase of the formulation. This serves as the priming dose, while the remainder is available for longer term trickle-release delivery which was consistent with the earlier CSLM fluorescence study and the cattle serology data.

Fig. 4.21 shows a very high degree of formulation stability, with the mean emulsion hydrogel diameter changing from approximately 0.2 to 0.4 microns over a five-month period. This suggests that storage of the vaccine formulation at room temperature is likely to have minimal effect on activity. However, this would need to be confirmed by animal inoculation studies.

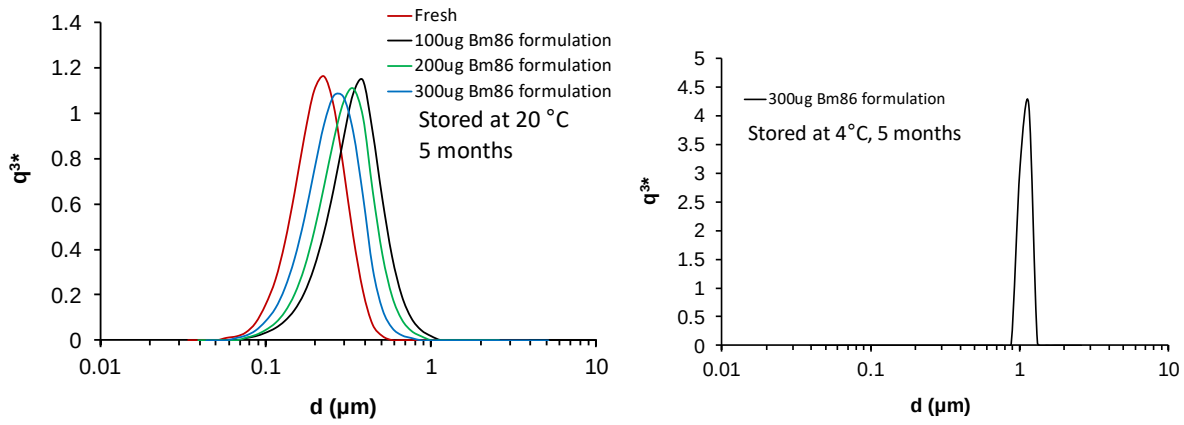

Fig. 4.21. Dynamic Laser Scattering (DLS) of freshly prepared sample of microhydrogel in emulsion formulation and samples stored for 5 months at 20 °C (RT) with Bm86 antigen loadings of 100, 200, and 300 µg per 3.5 mL dose. The corresponding emulsion size distribution when stored at 4 °C and returned to room temperature is given for 300 µg Bm86 loading.

4.3.2 Qualitative ELISA evaluation of the formulated Bm86 stability

Fig. 4.22 shows the phase separations observed following the extraction of the Bm86 from the vaccine formulations to determine if it had degraded over the five month incubation period.

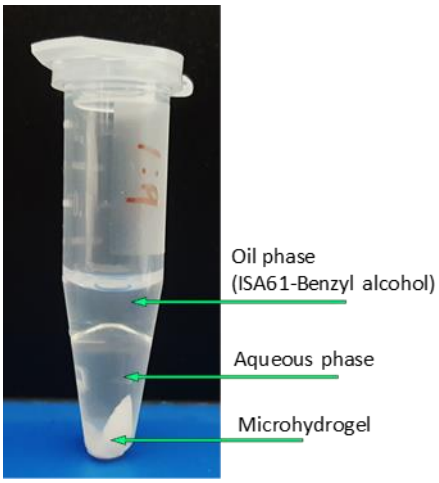

Fig. 4.22. De-emulsified microhydrogel in the emulsion formulation illustrating the free Bm86 in the aqueous phase and the bound Bm86 in the separated hydrogel pellet.

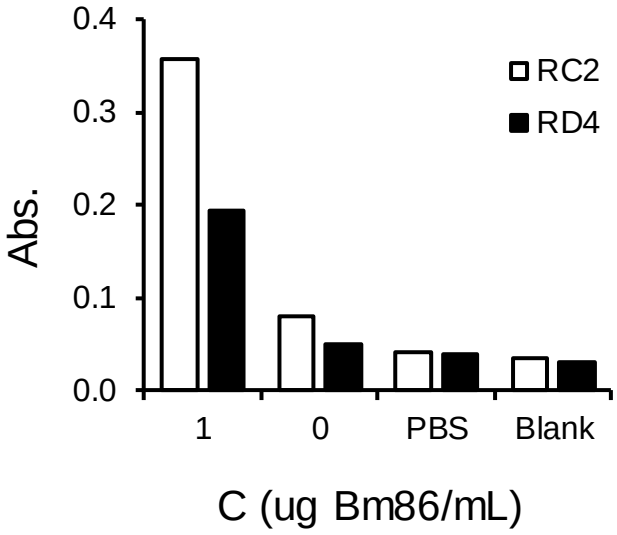

Fig. 4.23. The ELISA test results for Bm86 standards in blank bulk hydrogel syneresis liquid (containing traces of soluble biopolymer hydrogel) to RC2 and RD4 primary antibodies in comparison to the syneresis liquid from Blank bulk hydrogel and PBS.

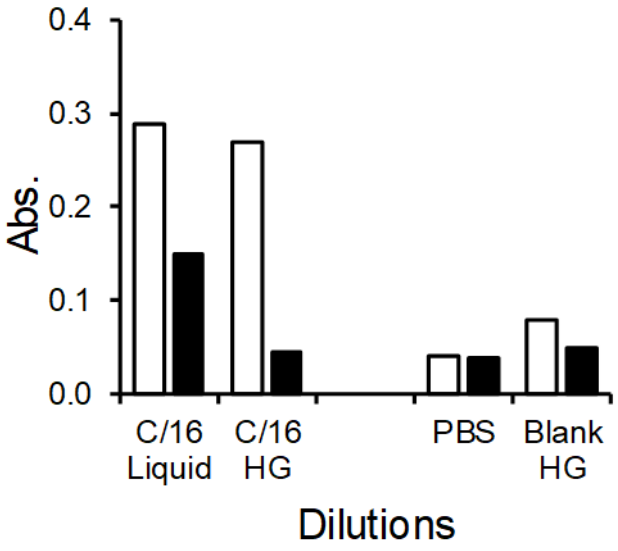

Fig. 4.24. The ELISA test results of free Bm86 extracted from microhydrogel in the aqueous phase of the microhydrogel produced by syneresis process (Liquid), and bound Bm86 in the microhydrogel released by resuspension of the pellet in blank bulk hydrogel syneresis liquid for 48 hours(HG). Samples were diluted 1/16 blank bulk hydrogel syneresis liquid. Illustrating the effect of the soluble hydrogel polymer components and the Montanide oil phase on the RC2 and RD4 complementary epitopes.

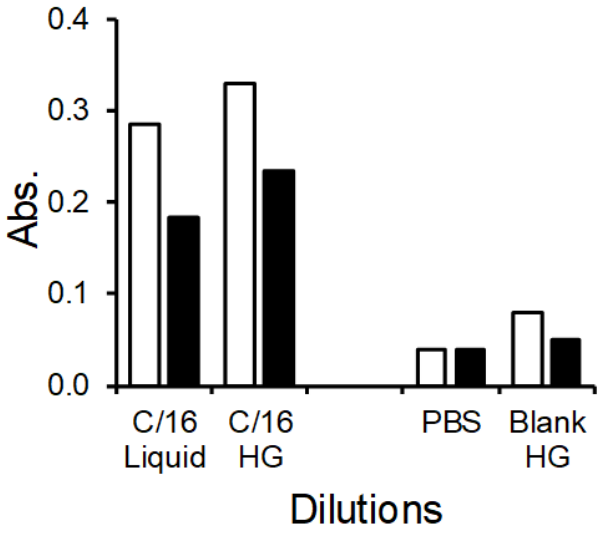

Fig. 4.25. The ELISA test results of the Bm86 extracted from a bulk hydrogel formulation, showing free BM86 in the aqueous phase of the bulk hydrogel formulation (liquid) and bound Bm86 in the bulk hydrogels which were released on resuspension of the pellet in blank hydrogel syneresis liquid for 48 hours (HG). Samples were diluted 1/16 in blank bulk hydrogel syneresis liquid. Illustrating the effect of the soluble hydrogel forming components only on the RC2 and RD4 complementary epitopes.

Figures 4.23 to 4.25 clearly indicate that quantitative ELISA testing of the Bm86 antigen within the microhydrogel formulations and the antigen extracted from these formulations undergoes interference from both residual traces of the biopolymer within the aqueous syneresis phase and the Montanide oil used as an adjuvant/emulsifier. Nevertheless, qualitative ELISA data provides significant information on characteristics of the Bm86 in the microhydrogel product.

5 Discussion

5.1 Strong and sustained immune responses from a single dose

Prior to the commencement of this project the lead polymer vaccine formulation had not been tested in cattle. Remarkably, the immune responses detected in the cattle immunised in this study were very similar to those detected in sheep in a previous study. Briefly, the responses in both species were characterised by an initial peak in antibody levels 28 days after primary immunisation

It is generally well accepted that Bm86 is a poor immunogen when used in conventional multidose vaccines as at least two injections are required to provide protection from larval infestations. This is evidenced by the need for regular booster injections to provide continued protection. The reasons for this poor immunogenicity is not readily apparent. Evolutionarily, cattle ticks and cattle are well separated on the “tree of life” and consequently the bovine immune system should be able to readily identify Bm86 as a foreign antigen. However, as the name suggests cattle are the primary host for cattle-ticks, this host/parasite relationship will have evolved thousands of years

Another reason why cattle-tick vaccination using a “hidden” antigen can be problematic is there is no natural boosting of the immune response following infestation. As Bm86 is located in the epithelia of the tick gut lining, the cells of the cattle immune system do not “see” it hence no boosting effect results. This is in contrast to other pathogens, e.g. viruses and bacteria, which establish sites of infection within the host and are therefore subjected to immune cell detection which can lead to re-stimulation of the immune system. As a result the only way to stimulate or restimulate the immune response is through delivery of additional doses. We believe that the NPV, replicates this process through either continued or periodic release of addition antigen/adjuvant complex, resulting continued boosting of the immune response.

Without doubt the major concern identified in this study was the swelling at the injection site of the NPV when the booster dose was administered. Although there was variability in the severity of these lumps, from none to severe, it would be clearly undesirably to have these develop in animals in large scale field immunisations. It is unclear what has caused these reactions. However, it seems likely that it may be to some of the components of the biopolymer formulation. While injection site reactions are not uncommon with conventional vaccines, where they do occur they are not as large nor persistent as observed in this study. Consequently, it would be of value to identify the component(s) in the current formulation linked to these overt responses. Once identified it may be possible to substitute the highly reactive component with a more inert component to eliminate/reduce the injection site swelling of the booster dose. Of course, any substitution of components may reduce strength and duration of the immunological responses resulting from vaccination.

The reactions to the booster injections presents a potential difficult situation for the in the field application of the single-dose formulation. If cattle remain on the property where they were vaccinated, and appropriate records are kept and maintained this may prevent revaccination of cattle. However, if cattle are sold the records of vaccination maybe no longer be associated with the animals, increasing the prospect of revaccination occurring. In addition, if the delivery system utilised in this project were deployed for other antigens of interest to the beef industry, then this

may inadvertently result in cattle being revaccinated with the NPV formulation, again resulting in injection site swelling. However, if the key actives of the vaccine, the Bm86 antigen and the ISA61VG adjuvant, are involved in the reaction site observed swelling, the negative effects of re-exposure maybe reduced or absent. Indeed, one way to investigate the underlying mechanism of the swelling would be to test the NPV formulation with an alternative antigen/adjuvant system in various combinations and evaluate subsequent responses. It is also possible that there is no need to use the complete NPV formulation for the booster dose, rather the Bm86/ISA61VG alone maybe be an effective secondary immunisation approach. However, this would create a vaccine with a multi-dose format where the safe application in the field would again require vaccination history to be linked to the animal.

In a pen trial setting the injection site swelling did not appear to have any effects on the performance of the cattle. No changes were observed in the behaviour of the cattle and no interventions were required. While one of the lumps did rupture, it only required constant monitoring and no intervention was required. Whether the lumps would remain benign in the field is open to question. In this less controlled environment, the risk of lumps rupturing and becoming susceptible to secondary complications (e.g. infection) would seem high.

5.2 Excellent protection of cattle tick larvae infection

The cattle immunised with the NPV formulation resulted in an excellent estimate of efficacy (76.9%). This level of efficacy is higher than the efficacy reported in studies in the literature were multiple doses have been administered and the cattle subsequently challenged at peak immunity (Hue et al., 2017; Jonsson et al., 2000). That we have achieved a similar level of efficacy 28 days and 56 days after peak immunity and primary immunisation, respectively, represents a significant step towards the development of a single dose cattle tick vaccine.

5.3 Stability of Bm86 within the NPV formulation

The Bm86 protein was not affected significantly when encapsulated and released from the chitosan-based hydrogel (i.e. the NPV formulation) when assessed using two Bm86 specific monoclonal antibodies (RC2 and RD4). This was confirmed with the bulk hydrogel produced in the absence of Montanide oil (which provided the greatest interference with the ELISA). These microhydrogel results show that the conformation and availability of Bm86 segments which complement RC2 and RD4 primary antibodies are maintained after formulation albeit to a lesser degree in the case of RD4.

From the results of the monoclonal antibody ELISA technique using RC2 primary antibody, about 40 - 50% of the Bm86 antigen is present free in the aqueous phase of the formulation available as the priming dose, which is consistent with the earlier CSLM fluorescence studies. The RD4 antibody showed a lower sensitivity to the antigen recovered from formulations which is consistent with serology evaluations at the University of Queensland laboratory. Overall these analyses confirm the formulation of the Bm86 antigen within the NPV formulation does not negatively affect its antigenicity. Moreover, the Bm86 was stable within these formulations for up to five months at 20°C

suggesting the formulation is resistant to degradation ambient temperatures. Further testing is required to determine the limits of this stability with respect to longer time periods and higher temperatures. The duration of dose stability is of key importance to vaccine manufacturers and distribution. While dose stability at ambient temperatures, can reduce the storage and shipping costs associated with vaccine distribution that should reduced the price paid by the end-users. Dose temperature stability can also crucial for the application of the vaccine in the field. As an example, if the vaccine is stable under ambient conditions then there is no need for a producer to ensure all animals are available for vaccination at the same time to ensure maximum utilisation of the available doses. Thus vaccine dose stability will allow producers more flexibility in the application of the vaccine that will drive adoption.

5.4 Project objectives

Initially this project had three objectives:

Objective 1: Determine the capacity of prototype single dose cattle tick vaccine formulations to induce immune responses in cattle.

This objective was clearly met, in both of the two trials conducted during the project, the groups of cattle immunised with the single-dose cattle tick formulation develop strong and sustained immune responses.

Objective 2: Determine the capacity of the lead single dose cattle tick vaccine to protect cattle from experimental infestations at peak immunity.

Objective 3: Determine the capacity of the lead single dose cattle tick vaccine to protect cattle from experimental infestations at six months post-vaccination.

Objective 2 and 3 were combined and collectively addressed. Timing of infestation was delayed to Day 56 post-immunisation, and excellent levels of protection from infestation were observed. The actual timing was delayed from the peak immune responses at Day 28, as at this point the responses were equivalent to or exceeded the responses in the TGL (SV) group. As it is well documented that TGL formulations can protect cattle from infestation, exceeding these responses strongly suggests the NPV immunise cattle would have been well protected at Day 28. However, as the overall immune responses drop to Day 56, infesting at Day 28 would not have provided any insights into what protection might be detected beyond this time point. By delaying the infestation to Day 56 it is now reasonable to conclude that cattle which respond well to the NPV are likely to be protected for around three months. As an extension to these objectives, animals from several of the groups in Trial 1 were maintained for one year to collect data on the duration of immunity. This information now provides insights into when additional infestation studies could be conducted to determine the duration of protection in a more rational and informative manner, rather than arbitrary time points. The further extension to these studies was to determine the responses of selected cattle to booster injections.

6 Conclusions/recommendations

While the results of the current project clearly demonstrate the feasibility of a single-dose cattle vaccine there are several areas where further research is required before the NPV would be ready

for adoption. Arguably, the most important area would be to determine the level of protection afforded by the NPV at different time points after immunisation. The current study determine the vaccine efficacy to be 76.9% when challenge commenced 56 days after immunisation. As the immune responses in the fluctuated over the year in which the trial 1 animals were monitored, it is not readily apparent when they would be protect or at what the level of vaccine efficacy would be at these time points. As a result, challenge groups of cattle regular time intervals would allow the immune responses to be correlated with protective efficacy. While establishment of this relationship would be expensive, once established it would expedite evaluation of the NPV as antibody levels could be used to predict efficacy. The relationship between antibody levels and protective efficacy would also simplify the evaluation of vaccine performance in field studies, where infestation cannot be controlled.

Another important parameter to be address is the variation in immune responses from animal to animal. Clearly, if the immune responses in animals classed as low-responders could be improved this would improve the performance of the vaccine in the field. One way to achieve this would be to try and standardise the delivery of the dose, perhaps using a dedicated subcutaneous injector. However, the variation in the immune responses of the cattle receiving the NPV vaccine were similar in the double formulation, suggesting the variation in responses may be more to do with individual animal responses rather than delivery method or route.

Finally, research is required to determine which of the vaccine components were responsible for the reactions at the sites of the booster immunisations. While, this project has established that the concepts which underpin the NPV formulation are sound, these reactions to the booster dose could be a barrier to adoption. Producers may not accept that the lumps are not negatively affecting animal performance. One approach to this would be to recommend animals not be given booster injections of the NPV formulation. The practicality of this approach is questionable given it would require the vaccination history of an animal be readily available when the decision to vaccinate or not is being made. A more practical approach would be to identify the component, causing the allergic response and then see if it can be replaced with a more insert substitute. Alternatively, it may be possible to reduce the amount of total material used per dose. Of course the key challenge in this approach is to ensure strength and duration of the resulting immune responses can still protect cattle from infestation.

Addressing the research described above would provide a clear pathway for the commercialisation and adoption of the NPV.

7 Key messages

The NPV vaccine evaluated in this project has the potential to revolutionise the control of cattle-ticks in the northern Australia cattle herd. The vaccine would provide producers with the capacity to reduce the production loses associated with this pest by protecting within the constraints of current industry practices such as infrequent mustering.

It has been estimated that if all of the cattle at risk of cattle-tick infestation could be reduced to the lowest levels of risk the economic return to industry would be $61M annually. An effective single-dose cattle tick vaccine could be a crucial component of an integrated pest management system to realise this return.

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9 Appendix A Summary of the experimental designs for Trial 1 and Trial 2

Table A9.1 Experimental design Trial 1 – Dose titration.

Day	Activity
0	1st injection: Novel Polymer Vaccine (NPV) 100μg Bm86 / 200μg Bm86 / 300μg Bm86 Tick guard like (TGL) 50μg Bm86 10 animals per group
28	2nd injection: TGL 50 Blood collected, all animals – antibody levels determined
56, 84, 112, 140	Blood collected, all animals – antibody levels determined
168	Five low responders removed from NPV_100 and NPV_200 groups Four low responders removed from NPV_300 Six low responders removed from TGL_50
Every 28d	Blood collected, all remaining animals – antibody levels determined
365	Blood collected, all remaining animals – antibody levels determined Booster injection: Six remaining NPV_300 animals All remaining animals euthanased & deep burial
Every 28d	Blood collected, all animals – antibody levels determined
560	Trial terminated Last remaining animals euthanased & deep burial.

Table A9.2 Experimental design Trial 2 – Protection study

Trial 2 – Vaccine efficacy	
-2	Record rectal temperatures
-1	Record rectal temperatures
0	Record rectal temperatures Blood collected all animals – antibody levels determined 1st injection NPV_300 TGL_50 24 animals per group (+ 24 unvaccinated controls)
28	Blood collected, all animals – antibody levels determined 2nd injection TGL 50
47	Blood collected, all animals – antibody levels determined
49	NPV 300 vaccinated (n = 16; randomly selected from 23highest antibody levels) & control animals (n = 8) moved to individual pens
56, 58, 61, 63, 65, 68, 70, 72, 75	Infest all animals with 4,000 tick larvae
77 - 97	Collect, wash & count dropped engorged female ticks daily
98	Blood collection (antibody levels determined), euthanasia and deep burial from the protection study animals
113	Blood collection (antibody levels determined), euthanasia and deep burial all remaining animals. Unexposed control animals (n=16) were sold.