finalreport

Animal Health and Welfare

Project code:	**B.AHW.0132**
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	Department of Employment, Economic Development and Innovation (DEEDI)
Date published:	**February 2010**
ISBN:	9781741915044

PUBLISHED BY

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Development of an improved frozen substitute for chilled tick fever vaccine for cattle

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Executive Summary

Tick fever has been an important disease of cattle in northern Australia since the introduction of the cattle tick more than a century ago. With industry support, the Queensland Government established the Tick Fever Research Centre, now Tick Fever Centre (TFC), to develop and produce a vaccine for control of the disease and, since 1966, more than 35 million doses have been supplied.

Traditionally, most of the vaccine supplied by TFC was made to order and issued chilled. However, existing production methods and service levels were not sustainable because of the high fixed costs and wastage as a result of the need for almost daily production, the short shelf-life and seasonality of demand. The need for an improved tick fever vaccine was identified in the MLA Project AHW 054A "Review of research needs for cattle tick control" (2005).

A frozen tick fever vaccine, now registered as "Combavac 3 in 1", has been available for about 30 years but, despite numerous refinements, demand for it has rarely exceeded ten percent of the annual total. Its particular appeal has been for the cattle stations processing large numbers of cattle, and those more remote locations where it is difficult to supply chilled vaccine and maintain the cold chain during delivery.

In an attempt to increase usage of Combavac, production of the chilled vaccine was ceased for two months during a period of low demand in 2005/06. This trial highlighted reasons for the poor adoption of Combavac; particular shortcomings were its very short (eight hour) shelf-life after thawing and the need to transport and store it in liquid nitrogen. Subsequent discussions between Queensland Government and cattle industry representatives led to agreement on the need for an improved frozen tick fever vaccine capable of replacing the chilled product. This project originated from those discussions and AgForce's support was crucial in the project team's successful application for funding.

A survey of tick fever vaccine users at the start of the project confirmed their high regard for the product and, not surprisingly, their continued need for an accessible, easy-to-use, affordable vaccine. The project's focus was therefore on the development of a frozen vaccine which closely emulated the chilled one in terms of efficacy, cost, packaging, transportation and shelf-life after reconstitution.

Glycerol is used as the cryoprotective agent in Combavac to protect tick fever parasites from the effects of ice crystal formation during the freezing process but has a negative effect on the organisms' viability after thawing. Changes to production procedures for the existing frozen vaccine (Combavac) greatly improved the pass rate of batches but attempts to extend the shelf-life beyond eight hours met with limited success. This vaccine must still be stored at ultra low temperatures in liquid nitrogen; storage in, for instance, a household deep freeze is not an option. Appeal for this vaccine will therefore remain mostly with the large and more remote cattle stations.

A major breakthrough in human blood transfusion services in recent years was the development of technology which allows blood to be frozen with glycerol and then for the glycerol to be removed after thawing. Using this technology, human blood can now be stored for years with recovery of up to 90% of red blood cells after thawing. Potentially, therefore, a frozen tick fever vaccine might also be thawed and reconstituted in the laboratory after removal of the glycerol, and issued in the same way as the chilled vaccine.

Exhaustive laboratory trials and field trials involving more than 1300 experimental cattle were conducted in attempts to adapt the human blood processing techniques to tick fever organisms. Of the three tick fever organisms, *Babesia bovis* is robust and remained viable for four days after bulk freezing, thawing and deglycerolisation but the yield was too small for the process to be suitable for commercial production of vaccine. *Babesia bigemina* was particularly vulnerable to deglycerolisation and very few organisms survived the process. *Anaplasma centrale,* the third tick fever organism in the vaccine, was successfully frozen, thawed and deglycerolised but it did not survive for four days in prepared vaccine.

It appears that bovine blood cells are more sensitive to the effects of glycerolisation and deglycerolisation than human cells. Presumably because of their complex internal structure and metabolism, tick fever parasites are also more susceptible to the stresses of freezing, thawing and deglycerolisation than the red blood cells within which they develop. The production of a standard chilled vaccine equivalent from a frozen concentrate was not commercially viable or cost-effective.

Concurrent trials with the standard chilled vaccine were conducted in order to provide a benchmark against which the efficacy of frozen vaccine could be measured. Results suggest that extension of the shelf-life beyond four days might be feasible. This would facilitate changes to the vaccine supply system, especially during the seasonal periods of low demand.

The immediate impact of the failure to develop a broadly acceptable, cost-effective frozen alternative for the chilled tick fever vaccine will be negligible. As there is no alternative supplier in Australia, the Queensland Government will continue to provide the service based mainly on supply of the chilled tick fever vaccine. According to a recent review using Queensland Treasury guidelines, the service is subsidised to an amount of $1.8m annually and there is no guarantee that the status quo can be maintained. Benefits of using the vaccine accrue almost 100% to industry, notably cattle owners in cattle tick infested parts of Australia, as well as those sending cattle to these parts and to some endemic countries overseas.

To ensure ongoing availability of a tick fever vaccine that meets industry needs in terms of affordability and efficacy, industry's support will be needed for the following initiatives:

- Further work on the shelf life of chilled vaccine - to facilitate a change in production schedules especially during periods of low demand, in order to ensure longer-term continuity of supply.
- Examination of alternative methods of production of *Babesia* vaccines - while the TFC calf-based vaccine production system complies with current Animal Welfare Codes, alternative methods of production need to be investigated in order to reduce reliance on animals.
- Improving protection afforded by the *Anaplasma* component of the tick fever vaccine - this need has been identified in other MLA-funded projects (DAQ107 and AHW.054A).
- Reducing the reliance on government subsidy by adjusting the vaccine cost structure.

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1 Background

1.1 Frozen tick fever vaccine – the early days

Tick fever was introduced into Australia more than a century ago and for many years it had a major effect on livestock industries in northern Australia. The history of the introduction and efforts to control the disease were described by Angus (1998)[1].

The need for more research on ticks and tick fever was recognised by the cattle industry and between 1962 and 1983 the Australian Beef and Cattle Research Committee (ABCRC)/ Australian Meat Research Committee (AMRC) contributed $1.4 million to the Queensland Department of Primary Industries (DPI, later DPI&F, now DEEDI) to assist in this research[1]. The bulk of the funds provided in the DAQ12 project "Studies on cattle tick, tick fever and tick control" was utilised at the Tick Fever Research Centre, now Tick Fever Centre (TFC),[2] and led to the Centre becoming the world leader in research on tick fever. Establishment of the Centre at Wacol was also made possible with ABCRC/AMRC funding. The Centre became operational in May 1966 and started producing tick fever vaccine almost immediately. Since then, the facilities have been upgraded extensively and, to date, more than 35 million doses of vaccine have been provided.

The main attribute of the vaccine is that it works; one "shot" provides good, lifelong protection against the three causes of tick fever: *Babesia bovis, Babesia bigemina* and *Anaplasma marginale.* This is not a claim that can be made for many animal vaccines. However, the vaccine does have some important weaknesses and an account of ongoing efforts to improve it, the milestones achieved and challenges faced during the first 30 years at Wacol was published in 1997 under the heading "Development of effective living vaccines against bovine babesiosis - the longest field trial?"[3]. The "trial" is continuing and current production methods[4, 5] are acknowledged world-wide as "best practice" [5].

Traditionally, all the vaccine supplied by TFC was prepared on demand and issued chilled. It was reliable provided it was transported and stored chilled, and used within days of manufacture. Some locations were difficult to reach while still maintaining the cold chain and the short shelf-life became a problem when scheduled vaccination programs were interrupted for any reason. While the product was very effective, existing production methods and service levels were not sustainable because of the high fixed costs and wastage as a result of the need for almost daily production, the short shelf-life and seasonality of demand. The need for an improved tick fever vaccine was identified in the MLA Project AHW 054A "Review of research needs for cattle tick control" (2005)[6].

Tick fever organisms have been frozen successfully in liquid nitrogen since the early 1970s[7], initially to preserve vaccine "seed stock". Later, this technology was used to produce frozen vaccine to meet a small demand in the Caribbean[8], Asia and South America, but also locally. This vaccine overcame the shortcomings associated with the chilled product's short shelf-life but contained dimethyl sulphoxide (DMSO), a cryoprotectant toxic for tick fever parasites at temperatures above 4^{o}C. It was used undiluted within 30 minutes of thawing.

In subsequent refinements of the vaccine, glycerol replaced DMSO as the cryoprotectant and allowed thawed vaccine to be kept for up to eight hours at room temperature[9]. This change was significant but still meant 'same-day' use of the vaccine. At the same time, a dilutable vaccine concentrate was introduced [10] and, in 2002, the vaccine was registered as "Combavac 3 in 1" with a minimum pack size of 25 doses. Since only one tenth of the vaccine's volume was frozen, the

impact on the cost of storage and transport (in liquid nitrogen) was significant. The major users of this vaccine were remote cattle stations in northern Queensland and the Northern Territory.

Despite these improvements to the frozen vaccine, the chilled product remained the most popular and comprised 88% of total demand in 2005/06.

1.2 Frozen vaccine trial 2005/06

Production of a chilled tick fever vaccine that is available year-round and made to order four days per week and in small batches has long been known to be unsustainable. To see if a frozen vaccine could replace the chilled one during summer months when the demand for vaccine was low, DPI&F conducted a trial from December 2005 to January 2006 during which only Combavac 3 in 1 was available. It was distributed from TFC and from depots at 16 veterinary practices spread throughout Queensland. Feedback from producers who used Combavac during the trial showed it had some major shortcomings and only 25% said they would use it again in its current form. Problems included:

- high cost of both the product and transportation
- need for storage in liquid nitrogen
- short post-thaw shelf-life (eight hours)
- minimum pack size (25 doses)
- need for thawing and dilution

Feedback from participating veterinarians suggested the storage, handling and reconstitution of Combavac were straight-forward but the post-thaw shelf life was too short. As their clients often wanted vaccine early in the morning before the veterinary surgery opened, a longer shelf-life would allow the vaccine to be reconstituted and collected the day before. They further commented that if the vaccine could be stored in a freezer, producers could take it home in frozen form and reconstitute it on site just prior to use.

1.3 Genesis of current project

As a result of the 2005/06 trial, further discussions were held between DPI&F and cattle industry representatives, including AgForce, on the need for an improved, cost-effective, user-friendly frozen tick fever vaccine capable of replacing the chilled product. This led to the development of the current project and the support of AgForce was crucial in the Department's successful application for funding. The AgForce Cattle Board was briefed on progress in the project in 2008 and again in 2009.

The original completion date of this project was 1 July 2009. However, loss of grazing land at Wacol due to changes in Government land use policy limited TFC's ability to do pen trials locally and increased the Centre's reliance on cooperator properties in tick-free areas. To allow for a final series of trials in 2009 and to fit in with the cooperators' mustering schedules, the project was extended to 30 January 2010 without an increase in funds.

1.4 Rationale for technical aims

1.4.1 Improve Combavac 3 in 1

As outlined in Section 1.2, Combavac suffered from a number of important deficiencies, including the need for storage in liquid nitrogen and a short (eight hour) shelf-life resulting in the need for "same day" use. One aim of the project was to address these deficiencies, and also the high cost of

production brought about by a high batch failure rate. In addition, we also proposed to look at the effect of route of administration on the vaccine's potency. A similar frozen tick fever vaccine produced in South Africa is administered only by the intramuscular (i/m) route as this is believed to reduce the proportion of cattle that fail to respond to vaccination. We prefer the subcutaneous (s/c) route for Combavac to minimise the risk of muscle damage.

1.4.2 Determine feasibility of removing glycerol from frozen vaccine concentrate after thawing to allow dispatch in chilled form

A major development in the storage of human blood in Blood Banks has been the long-term storage of red cells (RBCs) in the deep frozen state[11]. Using this technology (cryopreservation), blood can be stored frozen for many years [12] and for up to 21 days after thawing [13, 14]. Up to 90% of RBCs are recovered after thawing[13, 14]. Key to the success of this technique is glycerolisation of the blood (adding glycerol as cryoprotective additive or CPA to protect the RBCs from the damaging effects of ice crystal formation during the freezing process) and subsequent deglycerolisation (removal of the glycerol) after thawing. A special continuous flow centrifuge (e.g. a Haemonetics® ACP 215 machine) is used to achieve glycerolisation before freezing as well as deglycerolisation after thawing in closed, automated processes[15]. The technology has now been refined to the point where it is used internationally in blood transfusion services.

Glycerol is also used as the CPA in Combavac 3 in 1[9, 10] but removal of the chemical from the vaccine after thawing had never been attempted. It has a negative effect on the potency of thawed vaccine and its presence in the end product is the reason for the short shelf-life of frozen vaccine after reconstitution.

A major aim of the project was to see if frozen tick fever vaccine could be thawed, deglycerolised and issued as ready-to-use chilled vaccine and, if so, what the production capacity, cost of production and shelf-life of the vaccine would be. Important challenges we faced included the need to mimic the factors in blood which assist RBC metabolism (and also sustain the parasites), to prevent the swelling of RBCs during the deglycerolisation process [16] and to adapt cattle RBCs to a process developed using human RBCs.

1.4.3 Extending the shelf-life of chilled tick fever vaccine

The need for a replacement frozen product to address the weaknesses (cost, wastage) of the chilled tick fever vaccine has been outlined in previous sections. However, we accepted that, even if we succeeded with a frozen product, there may be an ongoing need for the chilled product in parts of the marketplace or at certain times of the year. From a consumer's point of view, the short (four day) shelf-life of this vaccine is a disadvantage, especially for those in remote areas and, from a manufacturer's perspective, the short shelf life is a major contributor to its high cost of production.

One aim of the project was therefore to also look at the feasibility of extending the shelf-life of chilled tick fever vaccine. Chilled vaccine infectivity also provided a benchmark against which we could measure the relative efficacy and shelf-life of the frozen vaccine products.

1.4.4 Improve laboratory techniques used to quantify tick fever parasites and assess viability

Organisms in raw material (donor blood) used to prepare tick fever vaccine are quantified using a method developed by Parker in 1973[17]. The accuracy of this direct counting method (counting of all the parasites in a set volume of blood) has been questioned in recent years and, to reduce variability

between counts, an alternative counting method (Traditional Method or TM) was assessed and validated.

During freezing and thawing, many tick fever organisms are killed[18, 19] and traditional counting methods are therefore of little value in quantifying the number of organisms in frozen vaccine. The only way to determine potency (viability) of these organisms in frozen vaccine has therefore been to inoculate cattle and then to monitor them for evidence of infection. This procedure is still the definitive test for vaccine potency but is expensive and time consuming.

The aim was to develop a staining method capable of differentiating between living and dead organisms that would provide a quick, inexpensive way of screening candidate vaccines and production procedures, and counting potentially infective organisms in vaccine concentrate after thawing. 'Vital' stains including fluorescein based dyes such as fluorescein diacetate (FDA) [20, 21] have been used for some time as indicators of cell viability. Similarly, red-fluorescent ethidium and propidium dyes which cross the membranes of dead or damaged cells, have been used as indicators of dead cells [22].

2 Project Objectives

a) Define producer requirements for tick fever vaccine by December 2006.
b) Reduce the batch failure rate in production of frozen tick fever vaccine from 50% to <20% by June 2009.
c) Develop cost effective and practical options for delivery of tick fever vaccine to producers by June 2009.
d) Determine the capability and cost-effectiveness of meeting producer requirements for tick fever vaccine by June 2009.

3 Methodology

3.1 General methodology

3.1.1 Alignment of trials with objectives

The project was designed to allow promising avenues to be explored and others to be abandoned if prospects for success were found to be small. To achieve the project's objective of meeting producer requirements, trials were designed with three ends in mind:

a) To improve the existing frozen vaccine (Combavac 3 in 1).
b) To develop a new frozen vaccine product as a means of storing blood to be reconstituted in the vaccine production facility and issued as a chilled vaccine.
c) To improve selected features of the existing chilled vaccine if progress in a) and/or b) appeared to be limited.

Chilled tick fever vaccine comprised almost 90% of the total vaccine sales and provided a standard against which we could measure potential infectivity and the shelf-life of the frozen vaccine product. The ideal result would be development of a frozen vaccine which can be stored in the domestic freezer and has the positive attributes of the chilled product.

3.1.2 Design of animal trials

Most of the animal trials were conducted on four co-operator properties in tick-free parts of Queensland. Subject to legal agreements with the DPI&F, the owners allowed access to the animals for vaccination and follow-up sampling. They also coordinated the mustering, handling and monitoring of the animals before, during and after vaccination.

We appreciated the great cooperation we received from the following producers, their families and staff:

John and Del Forrest	Oakleigh Grazing Co, Morven
Paul and Marina Wright	Woongarra Cattle Co, Taroom
Jim and Kaye Bock	Kilto, Wandoan
Bowhay Family Partnership	Buckinbah, St George

Conditions under which the trials were conducted are summarised in Appendix MS2-3. Some small pen trials were also conducted at TFC.

Only homebred calves were used on co-operator properties, most of them 8 - 10 months old and of homogeneous breed and size. Each calf was ear tagged (visual tags initially, later NLIS). With three tick fever organisms involved it was not possible to work out an ideal number of replicates so as many animals as were available were used. A group size of about 25 replicate animals per treatment allowed differences of 25% or greater in antibody positive rates between treatments to be detected at the 95% level of confidence.

The trial designs were as robust as we could manage given the limitations of field trials of this nature. The large number of treatments involved in each trial, hence the number of groups, and the limitations of the stock handling facilities on the properties precluded randomisation of the animals into groups before the start of each trial. As susceptibility to infection with tick fever is not known to be affected by animal factors other than possibly breed composition, randomisation was not considered important. Of greater importance was the need to treat all the replicates in each group within as short a space of time as possible given that the effect of each treatment on vaccine potency (infectivity of the tick fever parasites in the vaccine) was the primary objective of each trial.

Most animals were bled at the start of each trial to provide baseline data for the serological tests. The animals were then bled again about 70 days after vaccination and tested for the presence of antibodies to the tick fever parasites. Seroconversion was considered proof of vaccine potency.

All trials were conducted with the approval of an Animal Ethics Committee and the Australian Pesticides and Veterinary Medicines Authority.

3.1.3 Determining vaccine potency

Seroconversion of trial animals for tick fever organisms (*B. bovis*, *B. bigemina*. *A. centrale* and *A. marginale*) was determined by enzyme-linked immunosorbent assay (ELISA).

The method for *B. bovis* ELISA is essentially the same as the protocol used in Africa[23]; the *B. bigemina* cELISA is based on an immunodominant antigen now known as RAP-1[24], but with substantial modification to the method initially developed by Molloy et al[25]. The *A. centrale* cELISA is essentially unchanged from the published method[26] and the *A. marginale* ELISA is a commercially available kit (available from by VMRD®)[27].

For further descriptions and some information on the sensitivity and specificity of the tests, see Appendix 1 (this report).

To determine the potency of trivalent vaccine consisting of three parasites, a Disease Prevalence Factor (DPF) was used. It indicated vaccine potency weighted according to the percentage of field disease known to be caused by each of the parasites according to DPI&F diagnostic records. Approximately 80% of known field outbreaks are caused by *B. bovis*, with the remaining 20% split between *B. bigemina* (7%) and *A. marginale* (13%). The main reason for incorporating the DPF was the low sensitivity (hence high false negative rate) of the *B. bigemina* test.

3.1.4 Monitoring vaccine safety

There was little likelihood of vaccine reactions in the age group of cattle (<12 months of age) used in the trials. Nevertheless, the cattle owners were contracted to monitor the animals in the paddock for 60 days after vaccination (Appendix MS2-3). Veterinary support (private veterinarian or TFC veterinarian) was available should it be required.

3.1.5 Laboratory bench-top trials

A description of all the technical detail of the many laboratory analyses performed throughout the duration of the project is not warranted. However, substantial resources were directed towards examining the many factors involved in freezing, thawing and deglycerolising the frozen vaccine concentrates in order to improve both RBC and viable parasite recovery; and then to adapt any processes developed as manual techniques to automated processing using the Haemonetics® ACP-215 machine.

3.2 Define producer requirements for tick fever vaccine

3.2.1 Producer feedback following trial use of frozen tick fever vaccine

The 2005/06 trial during which only Combavac was supplied (see Background 1.2) was not part of this MLA project but feedback obtained during the trial was relevant to the development of the project and is therefore included here. Producers using vaccine during the trial were asked for their views on issues such as vaccine pickup and delivery, price, pack sizes, shelf-life, storage and handling, instructions and use of the vaccine. They were also encouraged to offer suggestions on how to improve the product.

Participating veterinarians were asked for their views on the vaccine and on the practicality of the service they provided.

3.2.2 Producer survey conducted in 2006

Aligned with Objective 2(a) of the project, a survey of tick fever vaccine users was conducted at the start of the project. It involved sending a questionnaire (Appendix MS1-2) to 3,369 recent users of the vaccine. The questionnaire was mailed to users on the DPI&F database or sent with vaccine to those ordering through agents. The survey was not designed to allow detailed statistical analysis but rather to allow DPI&F to get a general feel of producers' views of certain aspects of the delivery and use of tick fever vaccine that would have a bearing on the development and supply of a frozen product.

3.3 Improve "Combavac 3 in 1", the standard frozen vaccine

A series of trials were conducted in which Combavac was subjected to a range of treatments in an attempt to improve the shelf-life and the cold-chain requirements for transport and storage; and also to reduce the batch failure rate. Approximately 950 animals were used to assess infectivity and shelf-life of commercial and experimental Combavac batches in one small field trial at TFC and four large field trials on co-operator properties.

3.3.1 Reduce failure rate of batches of Combavac

Five animal trials were conducted during the project to assess and improve Combavac batch infectivity. The results of each trial was used to modify procedures for the next, with particular attention being paid to the method of glycerolisation, minimum acceptable parasite count for consistent infectivity, laboratory time spent processing and dispensing prior to freezing plus other relevant information obtained from studying alternative freezing protocols in the glycerolisation/deglycerolisation project.

One batch of vaccine was prepared using a new production method of adding glycerol and equilibrating each parasite separately before mixing, dispensing and freezing (Appendix 6, this report). This was then reconstituted using standard procedures and inoculated into two groups of cattle 8 and 18 hours later (Appendix MS3b-1, Groups 1 and 2). Because commercial batches of vaccine were involved, the main focus was on validation of potency and shelf-life for registration purposes, so no control vaccines were specifically prepared using the original method once initial trial work indicated acceptable infectivity with the new process. Following the successful outcome of the initial trial, 19 standard full commercial batches and three half batches (approximately 370,000 doses) of Combavac 3 in 1 were produced from 2007 to 2009, plus four more experimental batches using modified processes. The commercial batches were tested for potency eight hours after reconstitution (Appendices MS5-1, Table 1 and Appendix 3, this report, Tables 1 and 2) and the experimental batches after 8, 18, 24 or 36 hours.

To determine if other factors affected potency of Combavac, trials were also conducted to examine the effect of route of inoculation, dose volume and the number of parasites per dose (Appendix MS2-4).

3.3.2 Improve shelf-life of Combavac

Combavac has a registered shelf-life of eight hours after reconstitution. This "same day" use is inconvenient. Three field trials were conducted to assess the potency of experimental Combavac batches at various times after reconstitution (Appendices MS3b-1, MS5-1 and Appendix 3, this report). The limits of shelf-life were tested at approximately 24 and 36 hours for an experimental batch (EA43) that initially showed satisfactory infectivity at 18 hours after reconstitution. Variations to the method of processing, concentration of infected RBCs prior to freezing and storage in liquid nitrogen, concentration of glycerol in the cryopreservative and route of inoculation were also assessed with respect to infectivity at 8 to 36 hours.

3.3.3 Improve requirements for storage and transportation of Combavac

Two trials were conducted to compare the effect on potency if vaccine was stored in the laboratory on dry ice (-76^{o}C) instead of liquid nitrogen; alternatively, was stored in liquid nitrogen, then transported on dry ice and used on arrival or returned to liquid nitrogen on the property after transport on dry ice (Appendices MS2-4, Groups 2, 6 and 7, and MS2-5).

3.4 Feasibility of removing glycerol after reconstitution of frozen tick fever vaccine

3.4.1 Initial work

Based on human Blood Bank technology, a Haemonetics® ACP 215 machine and sterile docking device was leased from the company Medtel Australia® and used to prepare frozen cattle blood in two trials (Appendix MS1-4).

A series of bench top trials was conducted to investigate factors affecting the survival of bovine red blood cells (RBCs) during freezing and thawing and to find a way to successfully deglycerolise cattle RBCs. This quick screening tool allowed many potential variables to be addressed without the need to inoculate animals (Appendix MS1-5). Specific variables identified based on a review of the literature included natural fragility of uninfected and infected red blood cells; concentration of glycerol used as cryopreserving additive (CPA); concentration of red cells at time of freezing; cooling and warming rates during the freezing and thawing processes; composition of the recovery medium; deglycerolisation procedures; composition of the diluent used in deglycerolisation procedures; and the temperature of deglycerolisation (Appendix MS1-5).

3.4.2 Manual deglycerolisation of tick fever organisms

Based on results achieved in initial laboratory-based deglycerolisation trials, an infectivity trial conducted at TFC was designed to assess the effect of the concentration of glycerol used as CPA, the practice of concentrating the cells before freezing, the choice of buffer used, the speed of the cooling rate, the inclusion of sorbitol in the recovery medium and diluent, and the temperature of deglycerolisation. Thirteen groups of five animals were inoculated with manually deglycerolised vaccine and infectivity assessed (Appendices MS1-6 and MS2-1).

3.4.3 Adaptation of deglycerolisation procedures to allow automated processing of bovine blood with a Haemonetics® machine

Lessons learnt from initial bench trials, the pilot infectivity trial and subsequent field trials on factors affecting RBC survival before and during freezing were used in a series of four trials to modify the thawing and deglycerolisation procedures to allow automated processing of bovine blood with the Haemonetics® machine (MS2). Each trial involved the use of blood bags. Variables investigated included glycerol concentration, RBC concentration prior to freezing, expression of excess cryopreservative after thawing, buffer and diluent solutions, fragility of *B. bigemina*-infected RBCs, individual processing requirements of each parasite and dose/volume of blood required for satisfactory infectivity.

Notably the results from both the manual and automated deglycerolisation trials showed that each parasite had different requirements and needed to be dealt with individually. In the ensuing six field trials involving about 1300 animals, we therefore dealt with each parasite on its own. The trials involved incremental changes based on observations in preceding trials. Both manual and automated methods of processing were used.

Babesia bovis

Given the excellent infectivity observed in early studies, trials with *B. bovis* focussed on modification of the deglycerolisation process to suit an automated process (Appendices MS4-4, MS5-2a, and Appendices 2 and 5, this report), and clarification of dose and number of infected red cells to achieve satisfactory infectivity and shelf-life comparable with standard chilled vaccine (four day shelf-life).

Babesia bigemina

B. bigemina presented a unique case because initial results suggested a very poor rate of *B. bigemina*-infected RBC recovery during the deglycerolisation process, and very poor infectivity of deglycerolised vaccine. A specific work program focussed on improving RBC recovery and *B. bigemina* infectivity after the deglycerolisation process (Appendix MS3a-3).

Anaplasma centrale

Early studies suggested that infectivity of *A. centrale* started to decline 48 hours after reconstitution. Trials continued with the manual deglycerolisation process (Appendices MS4-5, MS5-2a, also Appendix 2 of this report), prior to attempting to automate the process using the Haemonetics® ACP-215 (Appendix 5, this report). In the latter Appendix, a small pilot trial is also described with a potential Anaplasma spp vaccine candidate (Dawn *A. marginale*)

3.5 Extending the shelf-life of chilled tick fever vaccine

Trials to validate an alternative method of quantifying tick fever parasites used in the production of tick fever vaccine (see 3.6.1 for more details) created an opportunity to reassess the survival of parasites in stored chilled tick fever vaccine. In one small trial at TFC (9 groups) and three major trials on co-operator properties involving 850 weaners (33 groups), observations were made on the viability of the three parasites with various permutations of blood and vaccine storage times and different parasite dose rates (Appendices MS3b-2, MS5-3, also Appendix 5, this report). Vaccine viability was assessed by testing the cattle for evidence of seroconversion about 70 days after vaccination.

3.6 Improving laboratory techniques used to quantify tick fever parasites and assess viability

3.6.1 Improved counting method for tick fever parasites

As part of the project, an alternative counting method (Traditional Method or TM) was assessed for use in trials and in routine production of tick fever vaccine. The number of parasites was calculated from the percentage of infected RBCs and the RBC count (Appendix MS3a-2). TM was initially validated in a pen trial using freshly made vaccine (Appendix MS3a-2) and later in a large field trial using blood and vaccine with different counts and storage times for every parasite (Appendix MS5-3).

3.6.2 Viability assay using vital stains

A series of trials were conducted using a range of yellow fluorescing ‘vital’ dyes and red counterstains to demonstrate the viability of parasites exposed to a range of stressors (Appendix MS3a-1).

4 Results and Discussion

4.1 General

4.1.1 Animals used

One initial small deglycerolisation trial (65 animals), one infectivity titration trial to validate chilled vaccine parasite dose rates (45 animals) and one small group Combavac batch infectivity trial

(20 animals) were conducted at TFC. Eight major field trials were conducted on the properties of three co-operating producers at Wandoan, Taroom and Morven, involving a total of approximately 3000 animals (from 300 to 450 animals per trial, with experimental group sizes ranging from 10 to 25 head). Producers were compensated to muster and work the cattle through the yards, and to monitor the cattle for signs of adverse reactions to the vaccine. This system worked well and allowed access to the cattle with minimal disruption to normal husbandry practices.

4.1.2 Determining vaccine potency

Reported sensitivities and specificities of the serological tests are noted in Appendix 1 of this report. Whilst the *B. bovis* test performs reliably, the *B. bigemina* test has less than optimum sensitivity and specificity; and interpretation of trial results for the *B. bigemina* component of chilled and frozen Combavac vaccine caused some difficulty. Results in some cases were inconsistent with expectations developed over many years of trials. The published reports of *B. bigemina* cELISA also note rapid drop off in antibody titres, within months in some cases[24]. A small routine infectivity test at TFC, including a newly developed real-time Polymerase Chain Reaction (PCR) test which detects *Babesia* DNA, will provide an opportunity to re-evaluate the sensitivity and specificity of the ELISA tests and decline in antibody levels over time. This will give some indication if the *B. bigemina* test is the problem or if there is indeed some issue with *B. bigemina* infectivity.

4.1.3 Vaccine safety

More than 3000 cattle were used on cooperator properties during the course of the project but no cases of vaccine reactions were reported. One animal broke its leg in one trial during vaccination and sampling, and was euthanased. Seven trial animals died on one property about 5-6 weeks after vaccination, but the cause was found to be clostridial disease and unassociated with the trial. Cattle not in the trial but in the same paddock as the trial animals and cattle in a different paddock also died.

4.2 Producer requirements for tick fever vaccine defined

4.2.1 Producer feedback following trial use of frozen tick fever vaccine

Notable problems identified by producers and participating veterinarians in the Combavac distribution trial of December 2005 and January 2006 included (see Background 1.2, this report and Appendix MS1-1):

- Cost of the product and transport
- Storage in liquid nitrogen
- Short post-thaw shelf-life (8 hours)
- Minimum pack size (25 doses)
- Requirement for thawing and dilution

4.2.2 Survey conducted in 2006

A total of 1,343 questionnaires (40%) were returned and the views of respondents can be found in Appendix MS1-3. In summary, the majority of respondents run commercial beef enterprises, order from 20 to 50 doses of vaccine at any one time and like the flexibility of having 10 and 20 dose packs so less wastage occurs. Most did not consider it important to have access to smaller pack sizes. One and five dose packs were only considered important by 18% and 30% of producers respectively.

The majority of respondents (73%) considered storage of vaccine in liquid nitrogen as not very useful but storage in a conventional freezer or fridge was deemed very useful (90% and 96% respectively). They also felt that the eight hour post-thaw life of the current frozen vaccine was too short. An extension of the post-thaw life to two days was considered to be useful by 66% of respondents whereas 80% considered a four or seven day life useful.

Feedback from respondents points to a high regard for DPI&F's tick fever vaccines supply service at the time and strongly recommended that the status quo should be maintained in terms of product accessibility, presentation and cost. The project's focus therefore had to be on the development of a frozen vaccine which closely emulated the current chilled vaccine in terms of efficacy, packaging, transportation and reconstituted shelf-life.

4.3 Improve "Combavac 3 in 1"

Improvements to infectivity, shelf-life and batch pass rates of Combavac 3 in 1 are closely intertwined, but the trial work for each is considered separately in the discussion below, despite considerable overlap.

4.3.1 Reduce failure rate of batches of Combavac

Prior to the start of the MLA project, variable infectivity led to an unacceptable batch failure rate (up to 50%) in pre-release infectivity testing. This increased the cost of supplying frozen tick fever vaccine and drove the need to find an effective solution. The cryopreservative containing glycerol is viscous, and incomplete mixing with a large volume of blood containing all three parasites prior to freezing was thought to be a possible cause of poor preservation of parasites during the freeze/thaw process. In an attempt to reduce the failure rate by improving glycerolisation during equilibration, the method was changed to allow blood collected from the different donors to be first glycerolised individually before being combined, mixed and dispensed (Appendix 6, this report).

Following a successful pilot trial with one batch of experimental Combavac vaccine (EA43) prepared using the modified method, 10 commercial batches (R043-R052) were prepared using the same method and tested for potency in 2008 (Appendix MS5-1, Table 1). The 'pass rate' of the batches was 70%, better than the old method of production, but still disappointing. Of the three batches which failed, two (R043 and R052) had the lowest *B. bovis* parasite counts prior to freezing (138 and 149 x 10^6 parasites/mL) while the third batch (R051) had a higher count (253 x 10^6) but the longest dispensing and freezing time during manufacture (>3 hours compared with 2-2.5 hours for most of the others).

B. bigemina infectivity was reduced in one batch, but this was not correlated with parasite count, nor with the time blood was stored before vaccine manufacture (in two batches, the *B. bigemina* blood was collected the day before and stored for 24 hours).

A. centrale infectivity was reduced in three batches, including R051 mentioned above (long dispensing time). No obvious reasons for the other two results were forthcoming.

A further nine full batches and three half batches (R053-R064), plus four experimental batches (EA73-EA76), were made in 2009 using the modified protocols (including a 1.75 fold increase in the minimum *B. bovis* parasite count to 350 x 10^6/mL, based on the TM count) (Appendix 3, this report). Infectivity of *B. bovis* was satisfactory at eight hours in 10 of the 12 commercial batches. One animal in each of the failed batches (R057 and R064) did not seroconvert to any of the parasites, and may have missed vaccination. Infectivity of *B. bovis* was reduced at eight hours in all four

experimental batches. In general, *B. bigemina* infectivity was satisfactory at eight hours for all experimental and commercial batches. The infectivity of *A. centrale* in the experimental batches was slightly reduced compared to the infectivity of the commercial Combavac 3 in 1 batches tested in both this trial, and previous batches.

The effect of other factors on vaccine potency, such as route of inoculation and dose, was also examined. In one trial, confounding results were obtained when *B. bovis* infectivity was improved by intramuscular injection, but *B. bigemina* infectivity declined, when Combavac was administered by the s/c and i/m routes 12-18 hours after reconstitution. In an attempt to clarify this, no difference was noted 18 hours after reconstitution in a second trial. (Appendices MS2-4, Groups 2 and 3, and Appendix MS3b-1, Groups 2 and 3). These results are in general agreement with those of an earlier trial where chilled vaccine was administered by the two routes. Increasing the dose volume from 2mL to 4mL while keeping the parasite numbers constant, did not improve vaccine potency. Potency was also not improved by a tenfold increase in the number of parasites per dose even though the potency of the standard vaccine was suboptimal (Appendix MS2-4, Groups 2, 4 and 5).

Conclusions:

- Modification of the production method led to a big improvement in the pass rate of vaccine batches.
- Inconsistencies in small scale manual production of experimental Combavac batches may have contributed to reduced infectivity of some trial batches (see Section 4.3.1).
- All future trials to test infectivity of commercial batches should preferably be done on co-operator properties using group sizes of 20-25 head, rather than five animal per group trials at TFC; this is a better test statistically and eliminates the risk of a batch failing if one animal fails to seroconvert to *B. bovis*.
- The purchase of a submersible magnetic stirrer is necessary to improve mixing during the glycerolisation process; this will reduce the risk of possible variations between batches caused by manual stirring.
- There were some differences in infectivity evident between parasites in the experimental batches. This suggests it may be worth producing experimental monovalent batches in order to clarify individual processing requirements best suited to each organism to ensure the maximum infectivity and shelf-life for each after reconstitution.
- Administration of Combavac by the i/m route did not improve potency of Combavac, nor did the size of the dose or the number of organisms per dose above the set minimum. To minimise the proportion of cattle failing to respond to vaccination, emphasis should therefore be on vaccine composition and production, not its administration.

4.3.2 Improve shelf-life of Combavac

Combavac has a registered shelf-life of eight hours after reconstitution. This “same day” shelf-life is too short to be of general use to most producers (see Background 1.2) while one of 18 to 24 hours (“prepare today, use tomorrow”) would render the product much more convenient in the field.

A series of trials were conducted during the course of the project to assess the potency of Combavac at various times from 8 to 36 hours after reconstitution. Initial trials with a single experimental batch (EA43) showed satisfactory infectivity of all three parasites at 18 hours (Appendix MS3b-1, group 2). However, when this same batch was reconstituted in a subsequent trial, *B. bovis* infectivity in particular was markedly reduced (<80%) by 24 and 36 hours (Appendix MS5-1), although *B. bigemina* showed excellent infectivity (>95%) and *A. centrale* infectivity was

approximately 90% at both time points. Four further experimental batches (EA73-EA76) were then prepared simultaneously from the same calf blood to reduce variation. These batches were designed to test a range of different pre-freeze processing procedures and reduced concentration of glycerol (2M - EA76) compared with the standard Combavac 3 in 1 (EA74) procedures. The different processing procedures compared concentration of the infected RBCs prior to (EA74) or after the addition of glycerol (EA73 and EA75), and also the concentration of RBCs during freezing and storage in liquid nitrogen (EA73-75). The results showed that potency of *B. bovis* could be maintained for up to 36 hours; that of *B. bigemina* was maintained to 24 hours but declining by 36 hours and, in general, that of *A. centrale* was declining by 24 hours (Appendix 3, Fig 1, this report). Except for *B. bigemina*, the six commercial batches tested at the same time showed superior infectivity to the experimental batches in almost all cases. There may also be some individual parasite preference for particular batch processing protocols – for example, *B. bigemina* and *A. centrale* infectivity fell away quite quickly in batch EA75 where the vaccine concentrate was frozen and stored in a very concentrated form (PCV 70%); whereas *B. bovis* infectivity was largely unaffected over the time tested.

Conclusions:

- With greater automation and further standardisation of production procedures, it may be possible to extend the shelf-life of Combavac beyond 8 hours, possibly to 18 hours. Some trials indicated satisfactory infectivity for all three parasites up to 18 hours, but the results were not consistent between trials. Such an extended shelf-life would allow reconstitution the day before use and would improve the appeal of this product and the feasibility of setting up depots in country centres.
- If reliable infectivity at a shelf-life beyond eight hours cannot be achieved, then there is every reason to keep a short life to ensure vaccine is used as soon as possible after thawing. This will reduce the possibility of cold storage requirements after reconstitution not being met (e.g. vaccines getting warm in eskies at yards, etc).
- Unless the shelf life can be extended, the appeal of Combavac will remain with the large, more remote cattle stations and demand for it is likely to remain at present levels (currently ~10% of total demand).
- It is possible that inconsistencies of small scale manual batch manufacture adversely affected infectivity. It will be essential that all future experimental vaccines be made in bulk quantities by the vaccine production team; especially now that there is a reduced need to test a wide range of options. This will better replicate the commercial frozen vaccine production system.
- More work is needed to confirm this shelf life over a range of vaccine batches and to determine the efficacy margin for each of the three parasites in the vaccine; and perhaps to determine individual processing preference for each parasite in monovalent vaccines. This work will be continued by DEEDI in 2010.

4.3.3 Improve requirements for storage and transportation of Combavac

Traditionally, Combavac has always been stored and transported in liquid nitrogen (-196^{o}C) and users identified this as a weakness of the vaccine during the trial conducted in 2005/06 (see Background 1.2).

To determine the effect of short and medium-term storage in dry ice, two trials were conducted. The results showed that vaccine potency 12 to 20 hours after thawing was not affected if it was initially stored in liquid nitrogen, then transported on dry ice and used on arrival or returned to liquid nitrogen until used. Longer term storage (six months) in dry ice, however, caused considerable loss of potency (Appendices MS2-4, Groups 2, 6 and 7, and MS2-5).

Conclusions:

- Because of the short shelf-life of reconstituted vaccine, maintenance of an ultra-low cold chain along transport routes will have to be maintained. Liquid nitrogen is the only long-term storage option that will retain satisfactory infectivity of the tick fever parasites.
- On-farm storage in, for example, a deep freeze, even for a short period, is definitely not an option.
- Combavac can be transported in dry ice and even kept at this temperature for several days without potency being affected. This provides an option for transportation other than use of liquid nitrogen containers, but not for longer term storage.
- Dry ice is not without its limitations – it is not cheap, it has a short finite delivery time of approximately 36 hours and is classified as a "Dangerous Good" with specific packaging and transport restrictions.

4.4 Feasibility of removing glycerol after reconstitution of frozen tick fever vaccine

4.4.1 Initial work

Initial trials using the Haemonetics® ACP 215 machine resulted in almost total lysis of cattle red blood cells (RBCs) during deglycerolisation. This suggested that bovine RBCs are more fragile than human RBCs when subjected to the osmotic and other stresses experienced during freezing, thawing and deglycerolisation. As a result, direct application of operating procedures and chemical solutions used to process human blood were unsuitable for production of tick fever vaccine (Appendix MS1-4).

We therefore redirected our focus to identify factors affecting the survival of bovine RBCs during freezing, thawing and deglycerolisation; to develop a small scale manual deglycerolisation process; and, finally to assess its effect on the viability of tick fever vaccine. Tick fever parasites grow inside RBCs and, while survival of these cells is not necessarily an indicator of parasite viability, it is assumed that RBC destruction will have a negative effect on parasite survival. The results indicated that RBC lysis could be significantly reduced, although not eliminated, by custom design of a process and solutions to better suit cattle RBCs (Appendix MS1-5). Importantly, in light of subsequent results, *B. bigemina* infected-RBCs seemed particularly fragile.

Correspondence with Dr Valeri, world authority on cryopreservation of human blood at the Naval Blood Research Laboratory in Boston, USA, indicated that we should follow precisely the human protocols[11], by which time we had already established that they did not work with cattle RBCs (see Results and Discussion 4.4.3). Human RBCs have been shown to have significantly reduced levels of haemolysis when stored at higher temperatures (-80^{o}C) using high concentration glycerol as the CPA (57% W/V) than when stored at lower temperatures (-150^{o}C) using less concentrated glycerol (29% W/V). He made no specific comments regarding the use of bovine RBCs, which in our system are stored at -196^{o}C using 3M or 4M glycerol (equivalent to 28% or 37% W/V respectively). Longer term high temperature storage had already been demonstrated to reduce parasite infectivity (see Results and Discussion 4.3.3). Peter Rolls, member of the project team, also visited the headquarters of the Haemonetics® Company in Boston for discussions with technical personnel. They indicated that while they often use cattle RBCs to validate processing and test protocols, unfortunately they had not ever used cattle RBCs to validate the automated glycerolisation and deglycerolisation process using the Haemonetics® ACP 215 machine. Whilst they were sympathetic to our cause, they were unwilling to commit any resources to researching the problem, even if it was

funded, unless there was some assurance of longer term sales of components and disposable items; that is, a commercial return even on time invested.

4.4.2 Manual deglycerolisation of tick fever organisms

In this initial pilot trial, infectivity of deglycerolised vaccine using various solutions and processing protocols varied from 40-100% (*B. bovis*), 0-80% (*B. bigemina*) and 20-100% (*A. centrale*); of particular note was that *B. bovis* and *A. centrale* infectivity of one group was 100% at 42 hours after reconstitution (Appendix MS2-1).

4.4.3 Adaptation of deglycerolisation procedures to allow automated processing of bovine blood with a Haemonetics® machine

Human blood is routinely frozen, thawed and washed to remove glycerol, using an automated process, with reported freeze-thaw RBC recovery rates of 93-95% and freeze-thaw-wash recovery rates of 85-88%. These figures are much greater than what we were able to achieve with bovine blood. Factors identified in bench top trials and pen trials (reported in MS2) as influencing RBC fragility and tick fever parasite survival were used as the basis to design further field trials; with sequential adjustments to process in subsequent trials, based on results.

Importantly, in field trials, no difference was detectable when vaccine concentrate was frozen in 5 mL tubes or larger volume bags; thus confirming that the manual tube process was a valid laboratory model for bags, and that automated vaccine production may be feasible. However, the marked variation in infectivity of the three parasites in initial field trials (Appendix MS3b-1) meant that for future trials each parasite was dealt with on an individual basis as a monovalent vaccine. Results for each parasite are outlined in the following sections; important results are highlighted whilst the specific changes and technical detail are not outlined in this discussion. The current glycerolisation and deglycerolisation protocols for *B. bovis* are outlined in Appendices MS5-2b and MS5-2c.

Babesia bovis

B. bovis proved to be highly infective three days after deglycerolisation and reconstitution from a frozen vaccine concentrate (Appendix MS3b-1). In subsequent trials (Appendix MS5-2a), deglycerolised *B. bovis* vaccine showed excellent infectivity (95-100% in most cases) at 3, 4 and 6 or 7 days post-inoculation, no matter the pre-freeze or post-thaw production process. The excellent infectivity of all groups still left some confounding factors for further investigation – 3M vs 4M glycerol; methods of glycerolisation; trivalent vs monovalent vaccine; the necessity for diluents other than standard chilled diluent, and several other issues.

Dose did appear to be more critical in frozen deglycerolised vaccine (Appendix MS5-2a). Chilled *B. bovis* vaccine (made with fresh blood) was still 100% infective at 0.1×10^7 parasites per dose ($1/10^{th}$ registered dose) after 14 days (Appendix MS5-3), whereas no group of deglycerolised frozen vaccine retained satisfactory infectivity at $1/10^{th}$ dose even at three and four days after reconstitution; this is in spite of an estimated pre-freeze starting dose greater than the minimum required dose of 1×10^7. The exception was trivalent vaccine where infectivity reduced both with time of vaccine storage and with dose dilutions but the starting dose was estimated to be below 1×10^7. It seems critical that deglycerolised vaccine be made with a dose estimate considerably higher than 1×10^7 to allow adequate efficacy margin (Appendix 4, this report).

Draft Standard Operating Procedures (SOPs) were developed for glycerolisation and deglycerolisation (Appendices MS5-2b, MS5-2c) to describe the best process developed by sequential progress over a number of trials.

It was not possible to accurately and consistently identify infected red blood cells in smears made from deglycerolised vaccine concentrate, and therefore vaccine dose was estimated by comparing RBC count in the deglycerolised product with that in the blood prior to freezing, and assuming that the percentage of *B. bovis*-infected red blood cells remained the same. The final trial of the project (Appendices 4 and 5, this report) aimed to validate the production SOPs and determine the production capacity (doses per donor calf) of a deglycerolised *B. bovis* vaccine, by clarifying dose required through a series of dose titrations, and thereby establish the feasibility of producing *B. bovis* vaccine in this way. Once dose was clarified, then constraints related to personnel, time required and volume of vaccine concentrate to be deglycerolised would be considered.

The results indicated quite clearly that to achieve satisfactory infectivity, a theoretical dose of approximately 1×10^8 parasites is needed, and infectivity falls off rapidly approaching a dose of 1×10^7 parasites (Appendix 5, this report). The number of infected RBCs to include in a dose of deglycerolised *B. bovis* vaccine is therefore approximately 10 times that included in standard chilled tick fever vaccine, in order to achieve satisfactory infectivity with some efficacy safety margin. With the parasitaemia of the donor blood used in this trial (549×10^6 parasites per ml), one bag was calculated to supply < 2400 doses, or less than the daily demand except during some of the quiet days in summer. At the minimum parasitaemia required in order to freeze blood (350×10^6 parasites per ml), one bag would only supply about 1500 doses.

Despite the high infectivity, poor recovery rates of RBCs (and presumably parasites) after the deglycerolised vaccine production process and inability to directly estimate parasite count at the completion of the production process were identified as potential constraints to the cost-effective production of a deglycerolised *B. bovis* vaccine. Nevertheless, if it proved not to be cost-effective compared with preparation of chilled *B. bovis,* if blood in excess of the requirements for weekly chilled vaccine production was frozen on a regular basis, a deglycerolised *B. bovis* vaccine might have been considered in the following situations:

- As a source of *B. bovis* parasites for addition to the standard trivalent chilled vaccine – either by using frozen vaccine stocks as a means of replacing a *B. bovis* donor calf in some weeks; or as a backup in the event that the chilled vaccine donor calf does not develop adequate parasitaemia (“fizzes”) or dies.
- As a means of quickly re-establishing sufficient parasitaemia for vaccine production, by intravenous injection of a large volume of deglycerolised vaccine concentrate, if the *B. bovis* donor calf fails or dies.

Babesia bigemina

B. bigemina infected-RBCs were significantly more fragile than those infected with *B. bovis* and *A. centrale* or cells from uninfected animals (Appendix MS1-5). We further demonstrated that, while *B. bovis* and *A. centrale* infected RBCs survived reasonably well during the deglycerolisation process, virtually all cells infected with *B. bigemina* lysed. A field trial confirmed that *B. bigemina* survival rates following the deglycerolisation process were very poor compared with the other parasites (<33% infectivity at 48 hours; <15% infectivity at 72 hours) (Appendix MS3b-1).

Increasing the RBC recovery in *B. bigemina* vaccine concentrate during freezing, thawing and deglycerolisation process did not increase the viability of deglycerolised vaccine. Improvements in

RBC recovery did not meet with any improvement in recovery of *B. bigemina*-infected RBCs (Appendix MS3a-3). In a further field trial, *B. bigemina* infectivity was also negligible (<33%) (Appendix MS3b-2).

One final trial (Appendix MS5-2a) demonstrated that *B. bigemina* deglycerolised vaccine had reduced infectivity similar to that of a monovalent *B. bigemina* Combavac frozen vaccine at 24 hours after reconstitution (<70%). Treatment of *B. bigemina* in the production of the vaccine may be quite different to that of the other two organisms. However, it was essential that improvements in the glycerolisation and deglycerolisation processes of all three organisms were fitted to an automated system; and so all work on a *B. bigemina* deglycerolised vaccine stopped prior to the last year of the project. Future work on frozen *B. bigemina* vaccine would focus on recovering the maximum number of parasites (whether extra- or intracellular) that will remain infective in the vaccine rather than focusing on preventing RBC lysis.

Anaplasma centrale

Initial pilot trials indicated a range of infectivity (20-80% at 18-42 hours) of deglycerolised *A. centrale* vaccine prepared using manual bench-top processes. Importantly, one group was fully infective at 42 hours after reconstitution (Appendix MS2-1, Group 3). In field trials (Appendix MS3b-1), *A. centrale* infectivity was reasonable at two days (81-95%), but reducing by three days (59-77%). Despite some changes to processing, marginal infectivity at three days (48-67%) was again confirmed at a subsequent field trial (Appendix MS3b-2).

Based on these results and also on laboratory trials aimed at improving the survival of *B. bigemina*-infected RBCs (Appendix MS3a-3), a number of parameters were assessed for their effect on survival of *A. centrale* (Appendix MS4-5). These parameters included the method of glycerolisation, centrifugation speed, changes to the deglycerolisation process, including centrifugation speed, addition of sorbitol to the buffer, post-thaw expression of cryopreservative and changes to the diluent. In laboratory trials, these changes did not appear to improve the recovery of infected RBCs.

In further field trials in 2008 (Appendix MS5-2a, Table 5), no *A. centrale* group achieved satisfactory infectivity at three days after reconstitution. In general, there was little advantage or inconsistent results associated with the method of glycerolisation prior to freezing, including leucodepletion and changing or adding extra serum to the diluent, or the method of post-thaw processing. Whilst we have been able to show retention of infectivity to seven days in chilled vaccine, once the dose is reduced we do not have the same efficacy margin as with *B. bovis* – that is, infectivity is lost more quickly as the dose is reduced and the same applies to deglycerolised vaccine.

Substantial benefit to infectivity in one group was gained with the addition of 20% serum to the standard chilled diluent. Results from trials incorporating both chilled vaccine and deglycerolised frozen vaccine suggested that the “buffering” and “nurturing” qualities of blood are important in the survival of *A. centrale* and/or *A. centrale* infected RBCs. Additive solutions used for storage of human RBC are focussed towards buffering against the toxic effects of RBC metabolism and providing the nutrients needed to sustain the RBCs for several weeks. RBCs in our vaccines are far more diluted and only need sustenance for a few days with, one anticipates, far less need for buffering capacity because of the greater dilution and shorter period of storage. However, sustenance is required for the metabolically active organisms inside the RBCs.

We know from trials with chilled vaccine work that *A. centrale* does not survive for extended periods without serum in the diluent (standard chilled diluent has 10% serum) and also needs >1 x 10^7 for satisfactory infectivity beyond the standard four day shelf-life. The final project trials were to clarify

the infective dose of deglycerolised *A. centrale* and the effect of serum concentration on infectivity (Appendices 4 and 5, this report). The only experimental *A. centrale* deglycerolised vaccine with satisfactory infectivity contained an estimate of greater than 1 x 10^8 parasites per dose in diluent containing 30% serum; that is, ten times the standard dose of parasites and three times the serum concentration in standard diluent. This was tested only to three days. Infectivity of a vaccine with greater than 1 x 10^8 parasites per dose, but with 20% serum in the diluent, did not achieve satisfactory infectivity at either three or four days. Improvements in infectivity in all groups were noted as dose and serum concentration increased.

Satisfactory infectivity of deglycerolised *A. centrale* vaccine to the equivalent four day shelf-life of standard chilled vaccine has yet to be demonstrated. Even to achieve satisfactory infectivity at three days, extra bovine serum (which is imported with certified health status from NZ) must be added to the diluent and at least 10 times the number of infected RBCs used for standard chilled vaccine must be incorporated into each dose. The same assumptions as with *B. bovis* were used to estimate dose, because of the difficulties encountered in direct parasite counts of the deglycerolised product.

Given the marginal infectivity obtained with *A. centrale* over a number of trials, the last trial of the project included some vaccine made from a field strain of *A. marginale*. This strain (know as Dawn)[28] has undergone some testing at TFC and has proved to be milder yet more protective against challenge strains than *A. centrale*. Deglycerolised vaccine was made from Dawn *A. marginale* monovalent concentrate (3M glycerol) and proved to have satisfactory infectivity at three days with high dose (and at 1/10^{th} titration) and 30% serum in the diluent; and at three days with high dose and 20% serum. The parasitaemia of the vaccine had not been calculated at the time of production, so no estimate of dose is possible.

4.5 Extending the shelf life of chilled tick fever vaccine

B. bovis remained viable in chilled vaccine for much longer than the current shelf-life suggests and, under conditions of a trial conducted in 2008, loss of infectivity was minimal in vaccine made from fresh blood and stored for 7 or 14 days (Appendix MS5-3, Table 2). These results show that the efficacy margin for *B. bovis* is considerable, even after 14 days, when vaccine is made from fresh blood. Standard vaccine at the limit of current registered shelf-life (four days blood storage and four days vaccine storage) retained infectivity at 1/10^{th} the standard dose without any allowance for "die-off" in stored blood (Appendix 5, Table 4, this report. This equates to approximately 1/45^{th} the number of parasites that would be included when vaccine is made from blood stored for four days. When this same vaccine was used at seven days after manufacture, infectivity was declining at the standard dose (without die-off allowance); that is, the efficacy margin was substantially reduced. Vaccine made with Day 1 blood and stored for seven days, also showed good infectivity at the standard dose, but efficacy was slightly reduced at 1/10^{th} the standard dose.

These results indicate considerable opportunity to extend the shelf-life of the *B. bovis* component in the current chilled vaccine. The shelf-life of *B. bovis* in vaccine would, however, depend somewhat on what is deemed an appropriate efficacy margin, and what the likely limits of blood storage would be if the number of days of manufacture per week was reduced in a new system of vaccine production, where vaccine was made less frequently but with a shelf-life beyond four days. Further trials are needed to clarify the infectivity and efficacy margin of *B. bovis* with various permutations of blood storage (from 1-4 days) and vaccine storage (from 4-7 days).

In the case of *B. bigemina* adequate viability was maintained for four days but dropped off when vaccine was stored for longer periods (Appendix MS5-3, Table 3). No further testing of *B. bigemina*

in chilled vaccine was performed in 2009, until some issues regarding the cELISA test were clarified in a smaller trial incorporating smear examination and PCR (see 4.1.2 above).

A. centrale infectivity was retained for seven days but evidently with no efficacy margin (Appendix MS5-3, Table 4). Also, vaccine diluent appeared not to support *A. centrale* as well as *Babesia* spp or alternatively, *A. centrale* is more susceptible once it is removed from blood and may need to be included at a higher dose in the vaccine to provide an adequate efficacy margin. Vaccine tested to the limits of current registration (vaccine made with Day 1 blood and stored for four days) was infective at quarter the standard dose, but infectivity was markedly reduced at 1/40th standard dose. The same vaccine stored for seven days showed poor infectivity at quarter the standard dose, even with the addition of extra serum to the diluent. Despite good infectivity at Taroom in 2008, vaccine made from Day 3 blood and stored for four days showed poor infectivity in this trial, even at standard dose with no allowance for die-off in the stored blood. When blood was stored for three days (to mimic one *A. centrale* blood collection per week), vaccine had poor infectivity at four and seven days (Appendix 5, Table 5, this report).

A. centrale vaccine appears to have a relatively limited efficacy margin (certainly less than tenfold) and limited survival beyond four days. Further trials should involve clarification of dose above 1 x 10^7 needed for satisfactory efficacy margin and examination of shelf-life at 5-6 days. *A. centrale* will be a limiting organism in any attempt to extend the shelf-life of the vaccine beyond four days.

To summarise, a seven-day shelf-life is clearly a realistic target for *B. bovis* and confirms observations made many years ago when the tick fever vaccine produced at Wacol consisted of *B. bovis* only and had a shelf-life of seven days. It is clear, however, that this target is not as readily achievable for the other two parasites and a more realistic aim for trivalent vaccine shelf-life lies somewhere between four and seven days.

4.6 Improving laboratory techniques used to determine parasite numbers and assess viability (MS3)

4.6.1 Improved counting method for tick fever parasites

The Traditional Method of counting (TM) gave variability within acceptable limits between specimens and between operators and was validated in a pen trial using freshly made vaccine (Appendix MS3a-2,Table 11) and in a large field trial with stored vaccine (Appendix MS5-3 Table 1). As a result, this counting method was introduced in the production process in 2008. In the case of *B. bovis,* the trials showed the efficacy margin for *B. bovis* was considerable when this counting method is used, even after 7 or 14 days. This has markedly reduced the likelihood that the continuity of supply of chilled vaccine will be interrupted, in that the amount of *B. bovis*-infected blood needed to provide reliable infectivity with an adequate efficacy margin has effectively been halved. Before this, a shortage of *B. bovis* infected blood was one of the main reasons for the occasional shortage of vaccine.

4.6.2 Viability assay using vital stains

Use of vital stains to differentiate between live and dead tick fever parasites worked well when used with fresh blood or when the parasites were killed rapidly with heat, as an example. However, after long-term storage of vaccine and after freezing, thawing and/or deglycerolisation procedures, the technique had serious limitations as the stains appeared to indicate evidence of biological activity which did not necessarily translate to viability of the vaccine and infectivity for the animal. Also, RBC membranes appeared to block the dead stains from entering the cells and potentially parasites in

them. As a result, few dead parasites were usually seen, only a reduction in the number of parasites staining “live’.

The results are a first for tick fever parasites subjected to the stresses of cryopreservation and were published in the international press[29]. However, this meant that rapid screening for the recovery of viable parasites from potential vaccine candidate processes was not possible in the laboratory and all infectivity tests had to be done in cattle.

5 Success in Achieving Objectives

5.1 Objective 1: Define producer requirements for tick fever vaccine by December 2006

This objective was achieved in terms of both producers’ views of the existing frozen tick fever vaccine (Combavac) and of tick fever vaccine in general. While they had a high regard for the service they received and were amenable to the concept of using a frozen vaccine, it was clear that a successful project outcome would be dependent on the development of a vaccine that is much more convenient to store and use than the current frozen one and preferably has attributes after reconstitution akin to those of the chilled vaccine in terms of shelf-life and infectivity.

5.2 Objective 2: Reduce the batch failure rate in production of frozen tick fever vaccine from 50% to <20% by June 2009

This objective was achieved. Modified procedures were used to produce 10 batches (about 180,000 doses) of vaccine in 2008 and resulted in a batch failure rate of 30% compared with 50% in the past. With additional minor modifications and in-process monitoring, the production procedure was streamlined further and nine batches and three half batches (~190,000 doses) were made and tested in 2009. The batch failure rate was 17%.

The reason why some batches still had reduced potency is not clear – in all cases, the failed batches were very close to having satisfactory infectivity. Despite the improvements, there are still some manual components in the production process which can lead to variability between batches and some equipment is now being purchased to further automate the process. The effect of these changes on the batch pass rate and on the post-thaw shelf-life will be assessed in 2010.

5.3 Objective 3: Develop cost effective and practical options for delivery of tick fever vaccine to producers by June 2009

This objective was partially achieved.

5.3.1 Improve shelf-life of Combavac after reconstitution

The registered shelf-life of Combavac is eight hours (effectively “same day” use). This short shelf-life is inconvenient to be practical and the aim was to achieve one of at least 24 hours (prepare today, use tomorrow). This target proved to be unachievable but one of 18 hours (although currently not consistent) may be, and would be a big improvement on eight hours. Work will continue on validation of the efficacy margin at 18 hours.

5.3.2 Improve storage and transport requirements of Combavac

Storage and transportation of Combavac in liquid nitrogen is expensive and cumbersome, and limits the usefulness of the product away from major centres. The aim was to look for alternative methods

of storage. Short-term storage and transportation in dry ice proved feasible and does provide other options for the distribution of this product. However, longer term storage on dry ice had a detrimental effect on vaccine potency and storage in a household freezer, a desired method according to producers, was certainly not an option.

5.3.3 Remove glycerol from frozen vaccine after reconstitution

B. bovis vaccine concentrate can be frozen, thawed and automatically processed to remove the glycerol, then issued as a chilled vaccine with a shelf-life of at least four days. It had been hoped after initial work that frozen and deglycerolised *B. bovis* might provide a number of options:

- as a source of *B. bovis* parasites for addition to the standard trivalent chilled
- as a backup in the event that the chilled vaccine donor calf does not develop adequate parasitaemia ("fizzes") or dies
- as a means of quickly re-establishing sufficient parasitaemia for vaccine production if the *B. bovis* donor calf fails or dies
- as a monovalent vaccine, the lone product available in the period of low demand.

However, the relatively fragile nature of cattle RBCs and the high theoretical dose of the parasites needed for satisfactory infectivity, mean that this is not a feasible option for routine vaccine production.

Currently, the post thawing shelf-life of deglycerolised *A. centrale* vaccine is too short for commercial consideration, and this technology could not be successfully adapted to process *B. bigemina*.

5.3.4 Improve shelf-life of chilled trivalent tick fever vaccine

The registered shelf-life of four days is too short for some of the larger producers in more remote regions and one of seven days was considered a practical and realistic target. An extended shelf-life of seven days is clearly achievable for *B. bovis* but not for the other parasites in the trivalent vaccine. Work is continuing on extending the shelf-life beyond four days. Even one of five days will be beneficial.

5.4 Objective 4: Determine the capability and cost-effectiveness of meeting producer requirements for tick fever vaccine by June 2009

Producer requirements cannot be met with improvements made to the frozen tick fever vaccines to date. To meet their expectations, it will be necessary to reconstitute frozen vaccine in the laboratory, remove the cryoprotectant and issue the vaccine chilled. While human blood can be processed in this way, it proved technically to be an unachievable goal despite intense efforts over three years and a visit to the equipment manufacturer in the USA. It appears that bovine blood cells and tick fever organisms in particular are very susceptible to the stresses of freezing, thawing and deglycerolisation.

Chilled tick fever vaccine has the capability of meeting producer requirements but problems with seasonal demand, wastage and high fixed costs were the principal motives for the development of this project.

6 Impact on Meat and Livestock Industry – now & in five years time

6.1 Impact on Meat and Livestock Industry – now

The aim of the project was to develop a cost-effective frozen tick fever vaccine that could replace the chilled vaccine and thus reduce production overheads but we were unsuccessful. The planned operational changes and cost savings expected as a result of a successful project will now not be achieved and further initiatives in this area need to be considered. The immediate impact of this for industry will however be negligible:

- Industry will continue to rely on chilled tick fever vaccine as it has done for the past 40 years.
- Most producers have adapted their management practices to accommodate the short shelf-life of the chilled vaccine.
- The Department of Employment, Economic Development and Innovation (DEEDI) is committed to continue providing the current service at least in the short term; however, using Queensland Treasury guidelines, a recent (2009) economic evaluation of the service showed it to be subsidised by the Queensland taxpayer to an amount of $1.8m per annum.
- To reduce the level of this subsidy vaccine prices will increase by 1.5% per annum above CPI until 2016/17; this is unlikely to deter many producers from using the vaccine, but will still fall well short of covering the costs estimated in the 2009 review.

6.2 Impact on Meat and Livestock Industry – in five years time

While the status quo can be maintained in the short-term there is no guarantee that this will still be the case in five years' time and changes will most likely be required much sooner. As there is no alternative supplier in Australia, closure of TFC is believed to be an unacceptable option for industry, despite the current financial pressures. It also now appears that the chilled vaccine will remain the principal product available to industry for the foreseeable future and this may have implications for the service provided in terms of cost and/or availability:

- Under guidelines and policies provided by Queensland Treasury, TFC is expected to "set user charges at a rate which accurately reflects the cost of providing the service".
- Benefits of the service provided by TFC accrue almost 100% to industry, indicating the policy of "beneficiary pays" could apply.
- To cover the current subsidy, it would be necessary to increase vaccine prices by 60%. This would almost certainly result in a consumer backlash and is unlikely to provide the required revenue.
- Year round daily production of chilled vaccine has mainly been for the convenience of industry and a change in production schedules during periods of low demand is proposed. The intention is to direct some TFC resources (facilities and staff) to other revenue-generating activities during these periods, in order to offset the high fixed costs of running the Centre. This may reduce the need for excessive price increases.
- The TFC calf-based vaccine production system, with stringent oversight by DEEDI Animal Ethics Committee, complies with current Animal Welfare Codes. However, DEEDI must look seriously at alternative methods of production used overseas in order to address implications of any future changes to animal welfare requirements.

7 Conclusions and Recommendations

7.1 Conclusions

Meeting producer expectations for a frozen tick fever vaccine that has the positive attributes of the chilled vaccine in terms of packaging, transportation and shelf-life was an unachievable goal.

7.1.1 Improving Combavac 3 in 1, the commercial frozen tick fever vaccine

- Modification of the production method led to a substantial improvement in the pass rate of vaccine batches.
- Combavac must be stored in liquid nitrogen; it cannot be stored in a household freezer. Distribution will at best be from depots, but in most cases, it will have to be transported to the property in a deep-frozen form (usually liquid nitrogen) for reconstitution on farm.
- The shelf-life of Combavac cannot currently be extended beyond eight hours (same day use); with greater automation and standardisation of production procedures, it may be possible to extend the shelf-life, possibly to 18 hours (prepare today, use tomorrow).
- Demand for this vaccine is likely to remain at current levels (~10% of total demand) and the appeal of Combavac will remain with the large, more remote cattle stations.

7.1.2 Removing glycerol cryoprotectant from reconstituted vaccine and issuing vaccine chilled

Production of a chilled trivalent tick fever vaccine from blood that is frozen, thawed and deglycerolised using human Blood Bank technology proved to be a noble but unachievable objective:

- *B. bovis* is robust and will remain viable for four days after bulk freezing, thawing and deglycerolisation but the yield is too small for production of vaccine using this process to be cost-effective.
- *B. bigemina* cannot be deglycerolised.
- *A. centrale* can be deglycerolised but the shelf-life is unacceptably short.

7.1.3 Improving chilled tick fever vaccine

- The need to find a cost-effective alternative was the principal motive for the development of this project; because of the failure to find a frozen alternative, the chilled vaccine will remain the principal product for prevention of tick fever for the foreseeable future.
- The chilled tick fever vaccine is highly effective. However, its perishable nature, high fixed costs of production, seasonal demand and liability issues ensure production is not commercially viable without government subsidy.
- A recent (2009) economic evaluation of the TFC service highlighted the need to make vaccine production more cost-effective as current charges do not accurately reflect the cost of production and deemed returns, according to Queensland Treasury guidelines. Removal of the substantial government subsidy might cause charges to increase by as much as 60%. Ceasing production of the vaccine was also listed as an option.
- Extension of the shelf-life of the chilled vaccine beyond the current four days may be feasible and this would facilitate changes in production schedules during periods of low demand.
- Other options to make the service more sustainable also need to be examined, especially in the period over late spring and summer when demand is traditionally low; options might include

seasonal reduction in the number of days that vaccine is produced each week or seasonal production of a bivalent vaccine (e.g. excluding *B bigemina*).

7.2 Recommendations

To ensure producers continue to have access to affordable, effective tick fever vaccine, the following issues need to be addressed in the production and supply of the chilled vaccine:

- The shelf-life needs to be validated with the view to extending it beyond four days to allow changes to be made to the production program. Without affecting continuity of vaccine supply, TFC resources (facilities and staff) could then be directed to other revenue-generating or Biosecurity Queensland service activities during periods of low demand and thereby help to offset the high fixed costs of running the Centre.
- The feasibility of producing vaccine from culture-derived *Babesia* spp needs urgent examination to reduce reliance on the calf-based production system. Calves will still be needed to produce the *Anaplasma* component of the vaccine but, while animal usage at TFC complies with current Animal Welfare Codes, alternative methods of propagating *Babesia* used overseas need to be investigated in order to address implications of any future changes to animal welfare requirements.
- The feasibility of replacing *A. centrale* with *A. marginale* (Dawn strain), a less virulent yet more protective alternative[28] needs to be investigated. This is especially important in view of the importance of anaplasmosis in *Bos indicus* genotypes in northern Australia as reported in earlier MLA projects (DAQ107 AHW 054A)[30 6 31]
- Consideration is given to changes to the vaccine production schedule and composition of the vaccine in seasonal periods of low demand.

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9 Appendices

9.1 Appendix 1: Serological tests for assessment of infectivity of tick fever vaccines

9.1.1 Test for *Babesia bovis*

The method for *B. bovis* ELISA was essentially the same as the protocol used in Africa [1] except 2% skim-milk powder was added to PBS containing 0.1% Tween 20 (PBST) instead of 5%. The other major change was the use of the peroxidise substrate TMB (Tetramethyl benzidine) instead of ABTS (2, 2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) requiring the absorbance to be read at 450nm, not 414nm. To control for inter-plate variation, known positive and negative sera were included in each plate. Test sera were then ranked relative to the positive control. ELISA absorbance results were expressed as a percentage of this positive control (percent positivity). For our purposes, non-reactive (NR) was <20% and reactive (R) was >40% with values in between considered "suspect".

9.1.2 Test for *Babesia bigemina*

The *B. bigemina* cELISA was based on the immunodominant 58 kDa antigen, now known as RAP-1[2]. The competitive inhibition ELISA originally developed used two monoclonal antibodies (MoAbs) (B9 and D6) directed against independent epitopes on the 58kDa antigen[3]. Since then, the antigen used in the ELISA is a 26 kDa peptide, encoded by a 360 bp fragment of the p58 gene, expressed in *Escherichia coli* and affinity purified. Use of the smaller antigen has dispensed with the need to use the MoAb D9 as a capture antibody and the antigen was coated directly onto the plate. This modified, yet to be published method is outlined below.

Test procedure:

i) The recombinant 26 kDa antigen was diluted in 0.1 M carbonate buffer (pH 9.6) to a concentration of approximately 2 µg/mL and 100 µL of that solution was added to each well of a 96-well microtitre plate. The plates were incubated overnight at 4°C.
ii) The solution containing any unbound antigen was removed and the wells were then blocked for 1 hour at room temperature by addition of 200 µL per well of a 2% solution of sodium caseinate in carbonate buffer.
iii) Following 3 brief rinses with 200 µL PBST (PBS containing 0.1% Tween 20), 100 µL of the diluted test and control serum (80 µL serum plus 20 µL 10% skim milk powder in PBST) was added and the plates were incubated for 30 min. at room temperature with gentle shaking.
iv) The plates were then washed with PBST (5 × 400 µL rinses). After the last rinse the plate was shaken for 5 minutes, then 100 µL of MoAb D6 diluted in PBST containing 2% skim milk powder was added to each well. The plates were then incubated at room temperature for 30 min. with gentle shaking.
v) The plates were washed again, as described in step iv. Next, 100 µL of peroxidase-labelled anti-mouse IgG diluted in PBST containing 2% skim milk powder was added and the plates were shaken for a further 30 min. at room temperature.
vi) The plates were washed again, as described in step iv. Next, 100 µL TMB peroxidase substrate was added to each well, and the plates incubated until the absorbance of the conjugate control wells (no serum) approached 1. At this point the reaction was stopped by the addition of 50 µL of 1M phosphoric acid and the absorbance was read at 450 nm. Positive and negative control sera were included on each test plate.

The percent inhibition (PI) for test sera was calculated relative to the conjugate control (PI = 100 – [100 × test absorbance/conjugate control absorbance]). Test cut-offs were usually determined for

each cattle mob based on pre-inoculation PI values so that NR was ≤ mean + 2 standard deviations (sd) and R was ≥ mean + 3 sd and PI values in between were considered "suspect". The specificity of the ELISA has been estimated at 97.0% and the sensitivity for detection of antibodies in experimentally infected cattle at 95.7% [3].

9.1.3 Test for *Anaplasma centrale*

The *A. centrale* cELISA was essentially unchanged from the published method [4] except that the MoAb used was now derived from culture and not ascites fluid. The percent inhibition (PI) for test sera was calculated relative to the conjugate control (PI = 100 – [100 × test absorbance/conjugate control absorbance]). Test cut-offs were usually set with NR PI values ≤ 50%, R PI values > 70% and PI values in between were considered suspect. Suspect results were not considered in calculating the DPF.

9.1.4 Test for *Anaplasma marginale*

The *A. marginale* cELISA was a commercially available kit (available from by VMRD) [5] and the manufacturer's instructions were strictly adhered to. The percent inhibition (PI) for test sera was calculated relative to the negative control (PI = 100 – [100 × test absorbance/negative control absorbance]). Test cut-offs were set with NR PI values < 30%, R PI values > 30%.

9.1.5 Test sensitivity and specificity

The table below shows the published sensitivity and specificity of the tests used for each of the three tick fever organisms. Note in particular the comparatively lower figures for *B. bigemina*.

Serological Test	Sensitivity	95% confidence limits	Specificity	95% confidence limits
ELISA for *B. bovis* (Molloy et al., 1998)[6]	100%	97.7, 100	99.4%	97.4, 99.9
B. bigemina (Molloy et al., 1998)[3]	95.7%	87.2, 98.9	97%	92.7, 98.9
Anaplasma centrale specific ELISA (Molloy et al., 2001) [4]	98.6%	91.6, 99.9	100%	87.4, 100

9.1.6 References

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9.2 Appendix 2: Combavac frozen vaccine and deglycerolised and reconstituted frozen vaccine: validation of infectivity and processing methods

9.2.1 Background

In studies previously reported (MS5), we demonstrated that *B. bovis* was a robust organism which could be frozen in 200mL bulk blood bags and remain infective for 7 days after thawing and deglycerolisation. However, clarification of dose and hence production capacity was still required in order to determine the feasibility and logistics of incorporating deglycerolised *B. bovis* into the vaccine production system. Options considered were to have deglycerolised *B. bovis* frozen vaccine concentrate as a back-up for donor calf failure to ensure continuity of supply; as a source of *B. bovis* organisms in trivalent vaccine in periods of low demand; or alternatively, in a complete revamp of the vaccine production process, as the source of parasites to supply a monovalent *B. bovis* vaccine in the periods of low demand.

Satisfactory infectivity had not been achieved beyond 2 days with deglycerolised *A. centrale* vaccine, nor had there been any assessment of *A. centrale* infectivity after bulk storage in bags and automated processing.

Whilst the incorporation of all three organisms into a deglycerolised frozen vaccine concentrate would have been ideal, the decision had been previously made that the parasites in deglycerolised vaccine would be dealt with as monovalent vaccines in order to be able to assess the individual requirements of each of the parasites. However, *B. bigemina* had proved impossible to freeze, thaw and deglycerolise without a devastating effect on infectivity and no further work was intended for this parasite in deglycerolised vaccine.

Three batches of commercial Combavac 3 in 1 were available for infectivity testing prior to release for sale.

9.2.2 Materials and Methods

Experimental cattle

Access to approximately 375 weaned cattle was secured on a cattle tick-free co-operator property near Wandoan; these were divided into 21 groups of 10 to 25 head each. Various permutations of deglycerolised *B. bovis* and *A. centrale* vaccines were tested including clarification of dose, storage of deglycerolised *B. bovis* stock solution, combinations of glycerolisation and deglycerolisation techniques for *A. centrale* vaccines, serum concentration in diluent and storage time after reconstitution (see Tables 1-5). Three groups were allocated to testing of Combavac 3 in 1 batches prior to release for sale (Table 6). The cattle were inoculated, and a sample bled and tested for antibodies to tick fever in June 2009; and then were bled and tested again in Aug 2009 (about 70 days later) to assess the rate of seroconversion in the different groups. Individual animals were inoculated either once with a trivalent vaccine, or on two occasions using monovalent vaccines made from different organisms. The development of antibodies to the vaccine organisms was taken as a definitive indication of vaccine potency.
The trial was conducted with the approval of an Animal Ethics Committee and the APVMA. The co-operator monitored the cattle for possible vaccine reactions and reported that none were detected. There were 11 deaths on the property, including 7 trial cattle, about 6-7 weeks after vaccination; 2 head died in the same paddock as the trial cattle and 2 others in a different paddock. Investigation by the local veterinarian and submission of samples to the veterinary diagnostic laboratory identified clostridial disease as the likely cause of the outbreak associated with a change to vaccination

practices on the property. There did not appear to be any association with the tick fever vaccine itself or with inoculation.

Experimental design

Blood containing the different organisms was collected from vaccine donor calves infected in accordance with routine vaccine production procedures. This blood was processed, glycerolised and frozen in bags (experimental *B. bovis* monovalent vaccine) or cryotubes (experimental *A. centrale* vaccine and Combavac 3 in 1 batches) as outlined below. The experimental vaccines were stored in liquid nitrogen, then thawed and deglycerolised also using methods outlined below. The vaccines were mainly reconstituted in standard chilled vaccine diluent containing 10% serum; several *A. centrale* vaccines were reconstituted in standard diluent with 20% serum. Some vaccine was made using deglycerolised *B. bovis* stock solution that had been stored overnight after processing and prior to reconstitution. Prior to inoculation, vaccines were stored for periods of 3, 4 and 7 days after reconstitution. Doses were calculated based on RBC count and parasitaemia of the donor calf blood and RBC count of the deglycerolised stock, assuming the same parasitaemia (that is, that uninfected and infected RBCs are lost at the same rate in the freeze, thaw and deglycerolisation processes). Several vaccines were also administered at $1/10^{th}$ and $1/100^{th}$ dose to evaluate the efficacy margin. Fresh *B. bigemina* and *A. centrale* were also included at the standard dose rate in one batch of deglycerolised *B. bovis* vaccine to assess any effect on infectivity.

Combavac 3 in 1 was reconstituted in standard frozen vaccine diluent following the prescribed method, and inoculated approximately 8 hours after thawing.

Glycerolisation Methods

Glycerolised frozen *B. bovis* vaccine (batch EA72) was prepared following the standard protocol for the glycerolisation described previously in Appendix MS4-4.

A. centrale iRBCs were glycerolised in two different ways. The blood for each of the batches of *A. centrale* iRBCs produced was also either filtered to remove leucocytes (leucofiltration) prior to glycerolisation or left unfiltered.

For the first glycerolisation method using concentrated blood, stored 1:1 with glycerol (*A. centrale* 4M glycerol in PBS with glucose (GPBSG) conc., 1:1), freshly collected infected blood was leucofiltered (or left unfiltered) then concentrated by centrifugation at 2429g for 15 minutes. The supernatant was aspirated and discarded. An equal quantity of 4M glycerol in PBS with glucose (GPBSG) pre-warmed to 37°C was added aseptically, and the mixture was incubated at 37°C in a water bath for 30 minutes. Final PCV of the glycerolised iRBC mix was about 35%. The blood was then transferred into 5ml Iwaki cryotubes, frozen for 15 minutes in the gaseous phase of liquid nitrogen before submersion and storage in liquid nitrogen.

For the second method of glycerolisation, an equal quantity of 4M glycerol in PBSG (pre-warmed to 37°C) was add to freshly collected leucofiltered blood (or unfiltered blood) and incubated at 37°C in a water bath for 30 minutes. After incubation the blood/glycerol mixture was centrifuged at 2429g for 15 minutes, and the supernatant aspirated and discarded, resulting in a final PCV of the glycerolised iRBC mix of approximately 70%. The concentrate was then transferred into Iwaki cryotubes and frozen and stored in liquid nitrogen. The glycerolisation protocols are summarised in Table 1.

Table 1: Glycerolisation procedure summary

Vaccine batch	Organism	CPA	Leuco-depletion via filtration	Centrifugation/ concentration of blood prior to glycerolisation	Centrifugation/ expression of excess CPA after glycerolisation	Final PCV (%) prior to freezing	Storage in Iwaki cryotubes or cryocyte bags
EA72	*B. bovis*	4M glycerol	Yes	No	Yes	78	200mL bags
EA68	*A. centrale*	4M glycerol	Yes	Yes	No	38.5	5mL tubes
EA69	*A. centrale*	4M glycerol	No	Yes	No	35	5mL tubes
EA70	*A. centrale*	4M glycerol	Yes	No	Yes	81.5	5mL tubes
EA71	*A. centrale*	4M glycerol	No	No	Yes	80	5mL tubes

Deglycerolisation Methods

Vaccine was deglycerolised following the standard method for tubes and bags (Appendices MS4-4 and MS4-5), except for variations shown in Table 2 for vaccine in bags and in Table 3 for vaccine in tubes. For the automated deglycerolisation in bags, the exposure buffer for each bag was 2M GPBSG + 1M sorbitol at 1x volume, with 2 dilutions using 0.5M sorbitol in PBSG (both at 2x volume), and 4 washes using 0.3M sorbitol in PBSG (volumes of 60mL, 70mL, 100mL and 150mL).

Table 2: Deglycerolisation procedure summary – vaccine stored in bags, automated deglycerolisation using Haemonetics ACP215 machine

Vaccine batch	Bag	Haemonetics protocol	Animal group no	Stock storage	Vaccine diluent	Fresh vaccine added	Vaccine storage (days)
EA72 *B. bovis*	1	**Protocol A** (16.667min equil, continuous addition of dilutions, shaker, 4 washes)	B3	0	Std chilled		7
			B6	1 day	Std chilled		7
			B12	0	Std chilled		7
			B15	0	Std chilled		7
			B18	0	Std chilled		7
EA72 *B. bovis*	2	**Protocol A** (16.667min equil, continuous addition of dilutions, shaker, 4 washes)	B1	0	Std chilled		3
			B2	0	Std chilled		4
			B4	1 day	Std chilled		3
			B5	1 day	Std chilled		4
			B7‡	0	Std chilled	*B. bigemina* & *A. centrale*	3
			B8‡	0	Std chilled	*B. bigemina* & *A. centrale*	4
			B9‡	0	Std chilled	*B. bigemina* & *A. centrale*	7
			B10*	0	Std chilled		3
			B11*	0	Std chilled		4
			B13	0	Std chilled		3
			B14	0	Std chilled		4
			B16	0	Std chilled		3
			B17	0	Std chilled		4

Note: The equivalent of 1mL of thawed vaccine was diluted to 20ml (Groups 1-9) or 1mL to 200mL (Groups 13-15) or 1ml to 2000mL (Groups 16-18)

* For groups 10 & 11 dose was calculated by parasitaemia to the equivalent of 1 x 10^7/dose

‡ *A. centrale* & *B. bigemina* infected blood added as per chilled vaccine dose rates (Groups 7, 8 & 9)

Table 3: Deglycerolisation procedure summary – vaccine stored in tubes, manual deglycerolisation

Animal group no	Vaccine batch	Exposure buffer	Equil time (min)	Centrifuge	Wash 1	Wash 2	Vaccine diluent	Vaccine storage (days)
A1	EA68	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A2	EA68	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A3*	EA68	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled	3
A4*	EA68	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled	3
A5	EA69	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A6*	EA69	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A7*	EA69	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled	3
A8	EA70	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A9	EA70	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.3M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A10	EA70	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled	3
A11	EA71	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A12	EA71	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.3M sorb in PBSG (1/10)	Standard chilled plus 20% serum	3
A13	EA71	2M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled	3

Note: * Group 3, 4, 6 & 7 – vaccine was centrifuged and excess CPA was expressed after thawing and before deglycerolisation
The equivalent of 1ml of thawed vaccine was diluted to 20mL (for batches EA70 and EA71) or 1mL diluted to 10mL (for batches EA68 and EA69).

9.2.3 Results and Discussion: Deglycerolised *Babesia bovis*

Results are found in Table 4. *B. bovis* vaccine was highly infective at 3, 4 and 7 days after vaccination at a theoretical dose exceeding 1 x 10^8, or ten times the inclusion rate of parasites in the standard chilled vaccine, when vaccine was made from freshly prepared stock solution. Infectivity was substantially reduced at the theoretical standard dose of 1 x 10^7 parasites; and even at approximately 2.5 x 10^7 parasites. Reduced infectivity was evident in the vaccine batches made from thawed and deglycerolised stock which had been stored overnight prior to reconstitution.

Deglycerolised *B. bovis* parasites incorporated into standard chilled vaccine made with fresh *B. bigemina* and *A. centrale* parasites were highly infective at 3 and 4 days, but showed slight reduction in infectivity at 7 days. The infectivity of the trivalent vaccine was satisfactory (as calculated by DPF %) at 3 and 4 days, but fresh *A. centrale* infectivity at standard 1 x 10^7 parasites per dose in the trivalent vaccine was substantially reduced at 7 days.

9.2.4 Results and Discussion: Deglycerolised *Anaplasma centrale*

Results are shown in Table 5. No groups had satisfactory infectivity at 3 days after reconstitution. In groups where the excess cryopreservative was removed prior to freezing and the infected RBCs frozen in a more concentrated form, infectivity was reduced, compared to groups where it was not removed at all, or only after thawing and prior to equilibration. This was in spite of the fact that the theoretical dose of the vaccines where the cryopreservative had been expressed prior to freezing was approximately 1.5 to 3 times higher. Addition of extra serum to the diluent also improved infectivity.

9.2.5 Results and Discussion: Combavac infectivity

Results are shown in Table 6. All groups showed satisfactory infectivity as assessed by the DPF%. *B. bigemina* infectivity was reduced compared to previous trials, especially in Batch R056. However, both Batches R055 and R056 had been tested in a small group of 5 animals at TFC earlier in the year: R056 had 100% infectivity for *B. bigemina* at that TFC trial. Infectivity of *A. centrale* at the larger field trial was satisfactory – one animal from each of batches R055 and R056 had failed to seroconvert in the 5 animal TFC trial.

9.2.6 Conclusions

Deglycerolised vaccine:

- The *B. bovis* dose required in the deglycerolised vaccine appears to be at least 10 fold higher than in chilled vaccine; and perhaps even greater for *A. centrale*. This requires clarification in the final trial of the project
- Increased serum concentration (>20%) in the diluent may be important to improve the infectivity of *A. centrale* vaccine at 3 days and beyond
- *A. centrale* vaccine is yet to be tested in bags

Combavac infectivity:

- Combavac infectivity was satisfactory, but *B. bigemina* infectivity was reduced compared to the trial results in 2007 and 2008 (Appendices MS3b-1 and MS5-1); no obvious reason for this was elucidated.

Table 4: Infectivity of *Babesia bovis* vaccine

Trial Group	*B. bovis* vaccine	Stock Storage (Days)	Fresh Vaccine Addition	*B. bovis* Calculated Dose	Dose Calculation Method	*B. bovis* Vaccine Storage (Days)	*B. bovis* Infectivity %
5	B1	0		24.4×10^7	Volume	3	100%
11	B2	0		24.4×10^7	Volume	4	94%
1	B3	0		22.4×10^7	Volume	7	100%
15	B4	1		24.4×10^7	Volume	3	90%
17	B5	1		24.4×10^7	Volume	4	83%
10	B6	1		22.4×10^7	Volume	7	90%
9	B7	0	*A. centrale & B. bigemina*	24.4×10^7	Volume	3	100%
16	B8	0	*A. centrale & B. bigemina*	24.4×10^7	Volume	4	100%
18	B9	0	*A. centrale & B. bigemina*	24.4×10^7	Volume	7	90%
6	B10	0		1.1×10^7	Dose based	3	68%
12	B11	0		1.1×10^7	Dose based	4	67%
2	B12	0		1.0×10^7	Dose based	7	65%
7	B13	0		2.5×10^7	1/10 Vol	3	85%
13	B14	0		2.5×10^7	1/10 Vol	4	70%
3	B15	0		2.3×10^7	1/10 Vol	7	69%
8	B16	0		0.2×10^7	1/100 Vol	3	50%
14	B17	0		0.2×10^7	1/100 Vol	4	50%
4	B18	0		0.2×10^7	1/100 Vol	7	0%

Table 5: Infectivity of *Anaplasma centrale* vaccine

Trial group	Batch	*A. centrale* vaccine	Wash 1	Wash 2	Vaccine diluent	Leuco-depleted	Pre-freeze Cryo Express	Post-thaw Cryo Express	Days storage	Calculated dose	*A centrale* infectivity %
5	EA68	A1	0.5M	0.5M	20% serum	X			3 days	8.8×10^7	68%
1		A2	0.5M	0.5M	10% serum	X			3 days	8.4×10^7	28%
2		A3	0.5M	0.5M	20% serum	X		X	3 days	4.4×10^7	82%
3 and 4		A4	0.5M	0.5M	10% serum	X		X	3 days	4.2×10^7	23/18%
10	EA69	A5	0.5M	0.5M	20% serum				3 days	8.8×10^7	60%
11		A6	0.5M	0.5M	20% serum			X	3 days	4.2×10^7	72%
12		A7	0.5M	0.5M	10% serum			X	3 days	4.2×10^7	44%
6	EA70	A8	0.5M	0.5M	20% serum	X	X		3 days	12×10^7	56%
8		A9	0.5M	0.3M	20% serum	X	X		3 days	12×10^7	17%
7		A10	0.5M	0.5M	10% serum	X	X		3 days	12×10^7	8%
15	EA71	A11	0.5M	0.5M	20% serum		X		3 days	12×10^7	22%
14		A12	0.5M	0.3M	20% serum		X		3 days	12×10^7	20%
13		A13	0.5M	0.5M	10% serum		X		3 days	12×10^7	22%

Table 6: Infectivity of Combavac 3 in 1

Group	Combavac Batch	Vaccine Storage (hours)	*B bovis* % R	*B bigemina* % R	*B bigemina* % R or Susp	*A centrale* %R	DPF %	DPF % With *B. bigemina* %R or Susp
19	R055	8	100%	80%	84%	96%	98%	98%
20	R056	8	96%	68%	72%	100%	95%	95%
21	R060	8	100%	72%	84%	100%	98%	99%
TFC 5 animal trial 2009								
	R055	8	100%	80%		80%	96%	
	R056	8	100%	100%		80%	95%	

9.3 Appendix 3: Routine and experimental Combavac 3 in 1 frozen vaccine: validation of infectivity and processing methods

9.3.1 Background

In studies previously reported, we demonstrated that all three tick fever organisms in one batch of experimental Combavac 3 in 1 remained infective to 18 hours (Appendix MS3b-1); but in a subsequent trial *B. bovis* in particular showed reduced infectivity at 24 and 36 hours, despite relatively satisfactory infectivity of the other two organisms (Appendix MS5-1).

Seven of 10 commercial batches produced in 2008 passed infectivity tests (Appendix MS5-1). Two of the failed batches were noted to have the lowest *B. bovis* counts prior to freezing and the other batch had the longest processing and dispensing times during manufacture (see Table 1a and 1b). As a result, the minimum *B. bovis* parasitaemia of the donor blood acceptable for the manufacture of Combavac 3 in 1 was raised by a factor of 1.75 to 350 x 10^6 parasites per mL, using the validated TM count (Appendix MS5-3).

In this 2009 trial, 6 new commercial batches and 4 batches of experimental vaccines manufactured using different protocols were tested for infectivity after thawing and reconstitution. The results of infectivity tests of recent commercial and experimental Combavac batches are shown in Table 1. Note that EA43 initially showed excellent infectivity at 18 hours after thawing, but did not maintain that infectivity through to 24 hours in a subsequent trial.

9.3.2 Materials and Methods

Experimental cattle

Access to approximately 450 weaned cattle was secured on a cattle tick-free co-operator property near Taroom; these were divided into 18 groups of 25 head each. Six groups were used to test commercial batches of Combavac at 8 hours and 12 groups to test 4 experimental batches at 8, 24 and 36 hours post-thawing. The cattle were inoculated, and a sample bled and tested for antibodies to tick fever in July 2009. All were bled again and tested in September 2009 (about 70 days later) to assess the rate of seroconversion in the different groups. Individual animals were inoculated once only – all the vaccines were trivalent. The presence of antibodies to the vaccine organisms was taken as a definitive indication of vaccine potency. A proportion of the animals were also bled again in November as part of a trial to assess Polymerase Chain Reaction (PCR) as a diagnostic tool.

The trial was conducted with the approval of an Animal Ethics Committee and the APVMA. The co-operator monitored the cattle for possible vaccine reactions and reported that none were detected.

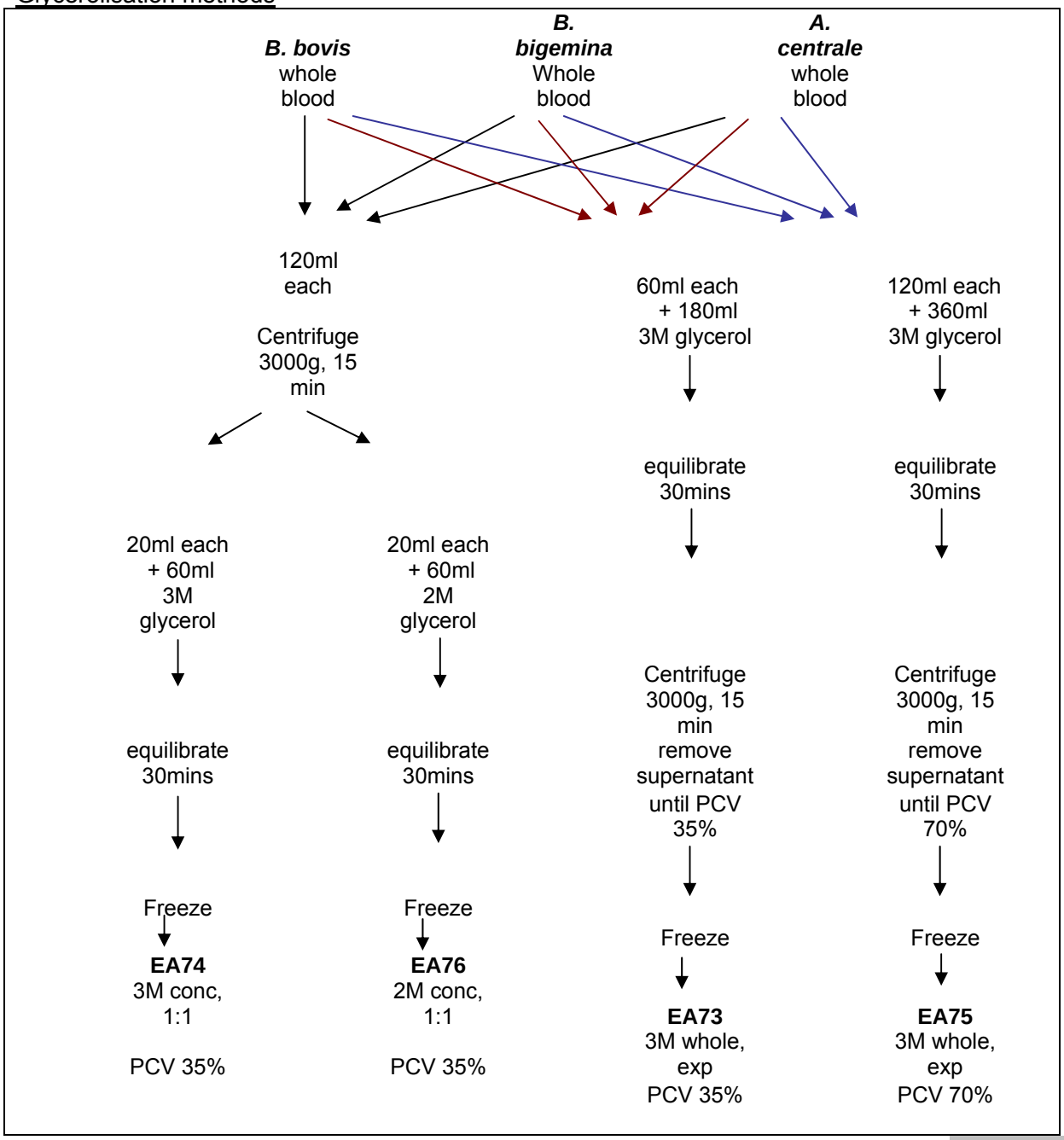
Experimental design

For the experimental vaccines (EA73-76), blood containing the different organisms was collected from vaccine donor calves infected in accordance with routine vaccine production procedures (see Table 3 – parasite counts for experimental and commercial Combavac batches in this trial plus experimental batch EA43). The experimental batches were prepared manually in small batches. This blood was processed, glycerolised and frozen in tubes with some modification to the SOPs. These batches were designed to test a range of different pre-freeze processing procedures and reduced concentration of glycerol (2M - EA76) compared with the standard Combavac 3 in 1 (EA74) procedures. The different processing procedures compared concentration of the infected RBCs prior to (EA74) or after the addition of glycerol (EA73 and EA75), and also the concentration of RBCs during freezing and storage in liquid nitrogen (EA73-75). The commercial and experimental

batches were stored in liquid nitrogen, and thawed according to standard protocols. The vaccines were mainly reconstituted in standard frozen vaccine diluent (containing 1.5M glycerol), except for the 2M experimental group (diluent contained just 1M glycerol). Prior to inoculation, reconstituted vaccines were stored for periods of 8, 24 and 36 hours.

Processing methods for each of the vaccine batches are outlined below:

Glycerolisation methods



Commercial Combavac batches were prepared and processed according to the SOP (9,6 Appendix 6, this report).

9.3.3 Results and Discussion

Results are found in Table 2 and Figure 1.

Babesia bovis

In general, the experimental *B. bovis* results in this trial were poor. EA74 was the standard Combavac equivalent and yet *B. bovis* infectivity at 8 hours was reduced compared to the infectivity of the other experimental batches. The generally reduced infectivity of *B. bovis* at 8 hours was surprising given the satisfactory infectivity of *B. bigemina* at 8 hours in all experimental batches. However, the infectivity of *B. bovis* at 24 and 36 hours was maintained in general at levels consistent with infectivity at 8 hours, suggesting that parasite die-off in that time was not great.

There might be some justification in trying the EA73 and EA75 style glycerolisation - that is, whole blood mixed with glycerol and the RBCs concentrated, rather than concentrating the RBCs prior to adding the glycerol. This, however, will use substantially more glycerol solution to process.

Infectivity of *B. bovis* at 8 hours in 5 of the 6 commercial batches was satisfactory. One animal in batch R064 did not seroconvert to any of the parasites, and may have missed vaccination.

Given the difference in infectivity between experimental and commercial batches, consideration should be given to the possibility that some of the reduced infectivity noticed in this trial is a function of processing small batches.

Babesia bigemina

In general, *B. bigemina* infectivity was satisfactory at 8 hours for all experimental and commercial batches, although slightly reduced in R059 and R062. Infectivity was also consistent to 24 hours, but declined by 36 hours after thawing.

Anaplasma centrale

The infectivity of the experimental batches was slightly reduced compared to the infectivity of the commercial Combavac 3 in 1 batches tested in both this trial and previous batches. Again, the possibility of an effect associated with processing of small batches should be considered. *A. centrale* infectivity at 24 and 36 hours after reconstitution declined more rapidly than for the other parasites. One commercial batch had an infectivity of only 80%; this batch still passed, given the requirement that the DPF% exceeds 95% and the infectivity of both *B. bigemina* and *A. centrale* exceed 80% (see Section 3.1.3, this report).

9.3.4 General conclusions

- Of the 12 batches of commercial Combavac made since the minimum *B. bovis* parasite count in blood for inclusion in frozen vaccine batches was raised to 350 x 10^6 parasites/mL, just 2 batches (R057, R064) have failed testing in 2009. In both these batches, one animal failed to seroconvert to all three parasites suggesting these animal missed vaccination
- We conclude that all trials to test infectivity of commercial batches should be done where possible on co-operator properties using group sizes of 20-25 head; this is a better test statistically and eliminates the risk of a batch failing if one animal fails to seroconvert to *B. bovis*
- We recommend that all future experimental vaccines should by made in bulk quantities by the vaccine production team to ensure that any inconsistencies of small scale manual batch

manufacture do not affect infectivity; especially now that there is a reduced need to test a wide range of options. This will also better replicate the commercial frozen vaccine production system

- We further recommend the purchase of a submersible magnetic stirrer to improve mixing during the glycerolisation process thereby eliminating possible variations in batches caused by manual intermittent stirring
- We consider it worth producing experimental monovalent batches in order to clarify individual processing requirements best suited to each organism to ensure the maximum shelf-life for each after reconstitution.
- All trials to test shelf-life of Combavac should be terminated at 18 hours, until reliable and consistent infectivity has been demonstrated at that time
- Despite the intentions to extend the shelf-life, if this is not reliable, then there is every reason to keep a short life to ensure vaccine is used as soon as possible after thawing to reduce the possibility that cold storage requirements after reconstitution are not met (eg vaccines getting warm in eskies at yards etc)
- The “Pass/Fail” criteria for infectivity of frozen vaccine batches (based on DPF% and individual parasite infectivity) should be reassessed

Table 1a: Commercial and experimental Combavac history

Group	Batch	Diluent	Time	B bovis %R	B bigem %R	A centrale %R	DPF%	Comments
St George 2006								
9	R023	Std	8 hrs	100	75	95	98	
10	R024	Std	8 hrs	100	63	95	97	
11	R025	Std	8 hrs	100	67	100	98	
Taroom 2007 (Appendix MS3b-1)								
1	EA43	Std	8 hrs	95	90	100	96	
2	EA43	Std	18 hrs	100	96	100	100	
3	EA43	Std	18 hrs	95	92	100	96	Intramuscular
Wandoan 2008 (Appendix MS5-1)								
1	R043	Std	8 hrs	78	96	91	81	B. bovis low count 138 x 10^6/mL
2	R044	Std	8 hrs	96	96	87	95	
3	R045	Std	8 hrs	100	100	100	100	
4	R046	Std	8 hrs	100	100	100	100	
5	R047	Std	8 hrs	96	91	100	96	
6	R048	Std	8 hrs	100	95	100	100	
7	R049	Std	8 hrs	96	100	100	97	
8	R050	Std	8 hrs	100	95	95	99	
9	R051	Std	8 hrs	83	96	91	85	Longest dispensing time
10	R052	Std	8 hrs	88	100	96	90	B. bovis low count 149 x 10^6/mL
11	EA43	2 yr frig	8 hrs	95	100	100	96	
12	EA43	2 yr RT	8 hrs	90	100	86	91	Diluent stored for 2 yrs at room temp
13	EA43	2 yr frig	24 hrs	78	100	91	81	Was infective to 18 hours in previous trial
14	EA43	2 yr frig	34.5 hrs	79	95	89	81	

TFC Small Trials 2009								
	R053	Std	8 hrs	100	100	100	100	5 animals
	R054	Std	8 hrs	100	80	100	99	5 animals
	R055	Std	8 hrs	100	80	80	96	5 animals – repeated at Wandoan 2009
	R056	Std	8 hrs	100	100	80	95	5 animals – repeated at Wandoan 2009
	R057	Std	8 hrs	80	80	80	80	5 animals; 1 animal NR for all parasites
	R057	Std	8 hrs	80	100	100	84	5 animals - intramuscular
Wandoan 2009 (see 9.2 Appendix 2, this report)								
19	R055	Std	8 hrs	100	84	96	98	
20	R056	Std	8 hrs	96	72	100	95	
21	R060	Std	8 hrs	100	84	100	99	

EA43 – standard 3M, conc, 1:1, glycerolised separately before combining (as per all commercial Combavac batches)

Table 1b Information summary on preparation of 10 batches of Combavac 3in1 in 2007/08

Batch No	Parasite	Donor No.	Days to prep	Count x10^6	PCV	*B bovis*	*B bigemina*	*A centrale*	No. Animals	DPFx	Preparation time (hours/min.s)	Delay	Disp time	Freezing time
R043	*B bovis*	8773	0	138	22.7	78%			23	**81%**	2h15	20min	1h	1;15
	B bigem	8739	0	168	29.1		96%							
	A centrale	8762	0	268	24.8			91%						
R044	*B bovis*	8858	0	244	20.4	96%			23	**95%**	2h20	60min	55min	1h
	B bigem	8764	1	283	34.7		96%							
	A centrale	8754	0	324	26.9			87%						
R045	*B bovis*	8870	0	283	21	100%			24	**100 %**	1h18	25min	1;05	1h
	B bigem	8769	0	185	30.1		100%							
	A centrale	8866	0	635	27.7			100%						
R046	*B bovis*	8851	0	366	24.2	100%			24	**100 %**	2h05	40min	1;05	1;30
	B bigem	8864	1	325	28.7		100%							
	A centrale	8846	0	452	28.9			100%						
R047	*B bovis*	8883	0	277	23.8	96%			23	**96%**	3h15	60min	1;00	1;15
	B bigem	8849	0	102	33.9		91%							
	A centrale	8865	0	148	31.5			100%						
R048	*B bovis*	8879	0	201	21.9	100%			22	**100 %**	1h45	1;10	45min	1;10
	B bigem	8860	0	412	27.8		95%							
	A centrale	8878	0	428	27.5			100%						
R49	*B bovis*	8899	0	192	28.8	96%			24	**97%**	2h30	15min	1;15	1;15
	B bigem	8875	0	72	33.4		100%							
	A centrale	8910	0	294	30.9			100%						
R50	*B bovis*	8915	0	172	29.4	100%			21	**99%**	1h50	35min	45min	1;15
	B bigem	8884	0	193	31.9		95%							
	A centrale	8911	0	125	20.2			95%						
R51	*B bovis*	8920	0	253	25.1	83%			23	**85%**	2h20	25min	1;40	1;30
	B bigem	8894	0	105	28.7		96%							
	A centrale	8926	0	212	28.3			91%						
R52	*B bovis*	8928	0	149	25.1	88%			25	**90%**	1h25	50min	65min	1;30
	B bigem	8904	0	209	27.7		100%							
	A centrale	8922	0	280	28			96%						

Table 2: Taroom 2009 results

Group	Batch	Diluent	Time	Bovis	Bigem	Cent	DPF%	Comments
7	EA73	Std	8 hrs	92	96	88	92	
11	EA73	Std	24 hrs	92	96	80	91	
15	EA73	Std	34.5 hrs	100	84	68	95	
8	EA74	Std	8 hrs	76	96	88	79	
12	EA74	Std	24 hrs	88	100	88	89	
16	EA74	Std	34.5 hrs	84	92	80	84	
9	EA75	Std	8 hrs	88	100	88	89	
13	EA75	Std	24 hrs	96	84	56	90	
17	EA75	Std	34.5 hrs	96	68	52	88	
10	EA76	Std	8 hrs	92	96	88	92	
14	EA76	Std	24 hrs	92	92	72	89	
18	EA76	Std	34.5 hrs	84	76	76	82	
1	R058	Std	8 hrs	100	92	80	97	Note *A. centrale* result
2	R059	Std	8 hrs	100	92	100	99	
3	R061	Std	8 hrs	96	96	88	95	
4	R062	Std	8 hrs	96	88	96	95	
5	R063	Std	8 hrs	100	96	100	99	
6	R064	Std	8 hrs	92	92	88	92	1 animal NR for all parasites

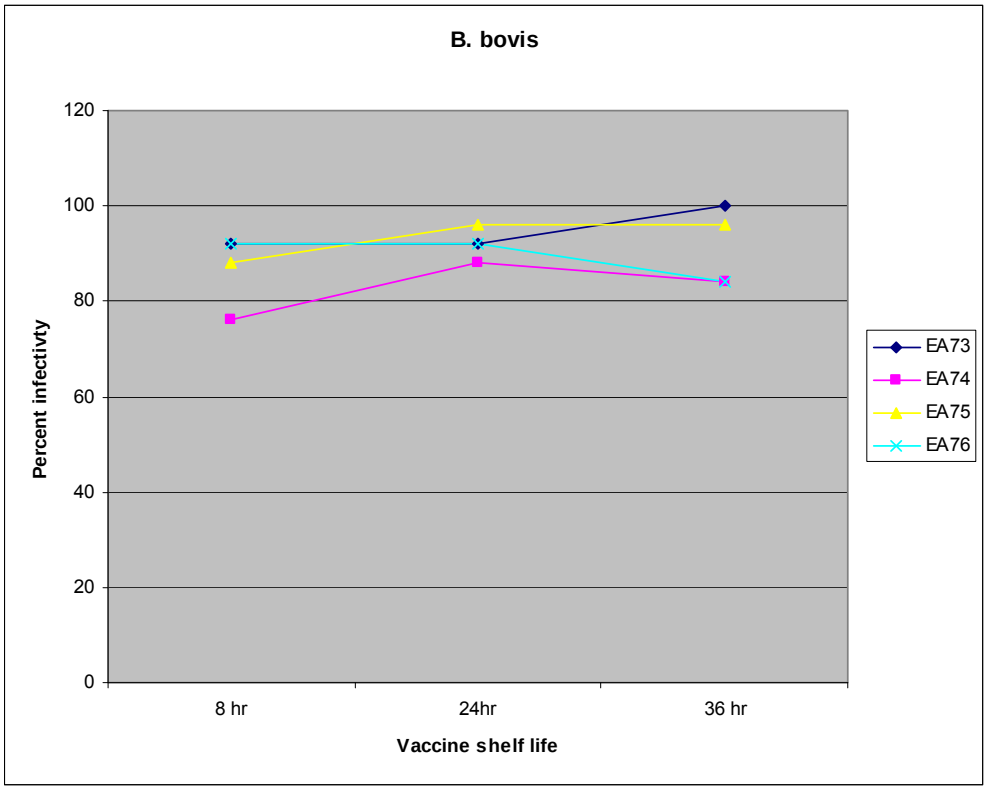
EA73 – 3M whole, expressed, PCV 35%
EA74 – 3M conc, 1:1, PCV 35% (standard Combavac 3 in 1 equivalent)
EA75 – 3M whole, expressed, PCV 70%
EA76 – 2M conc, 1:1, PCV 35%

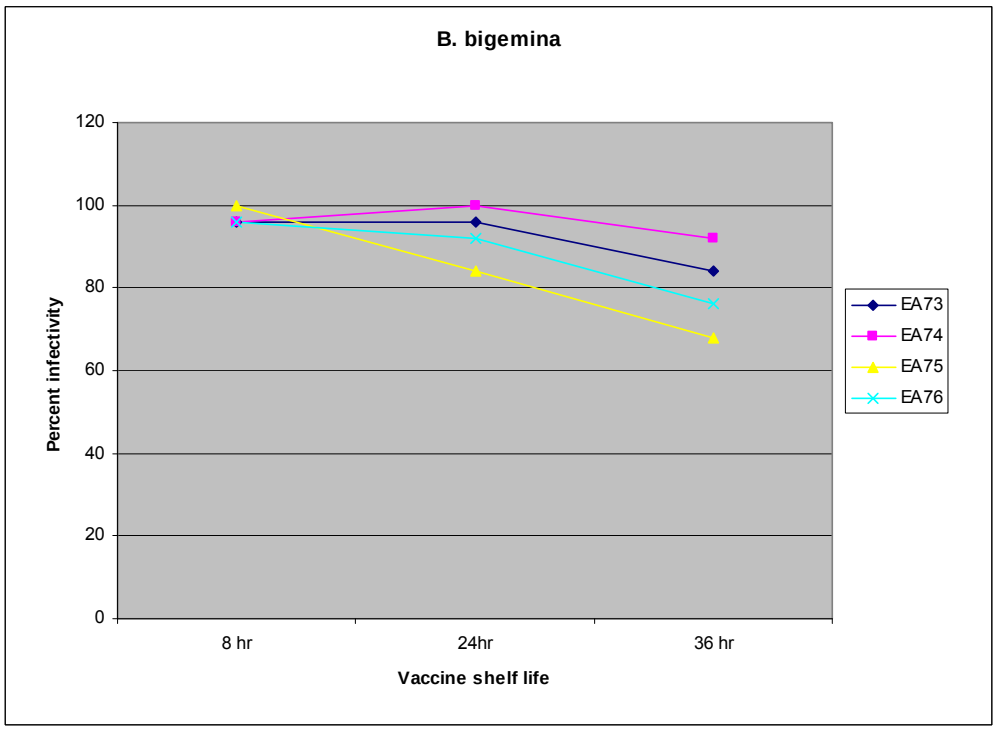
Table 3: Experimental and commercial Combavac batch – parasite counts for donor blood

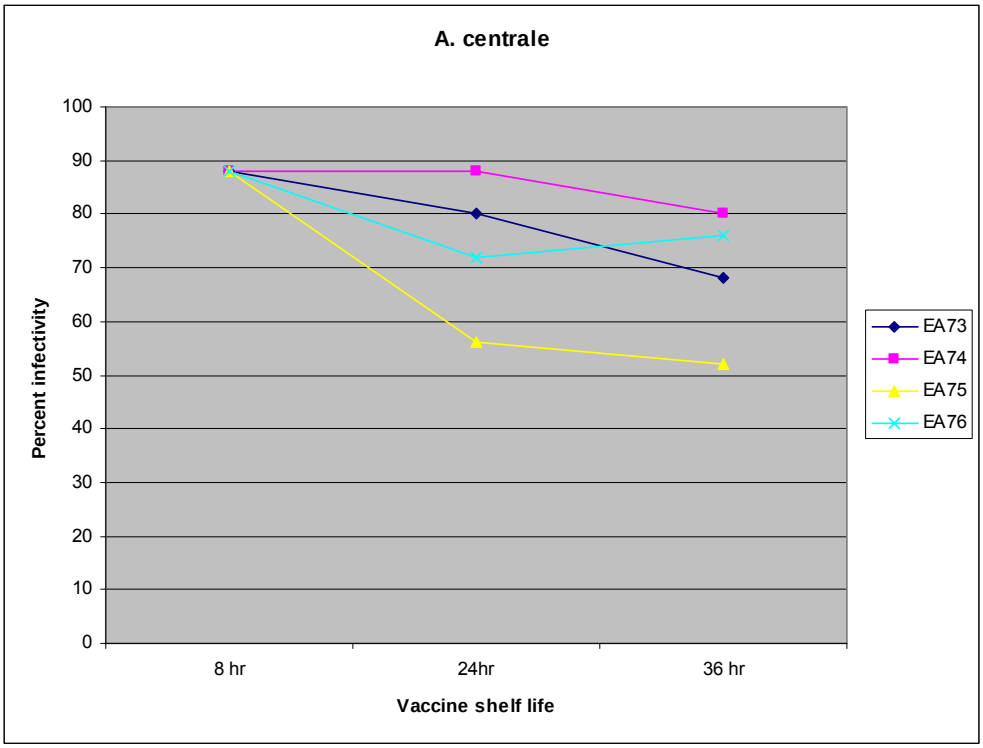
	Babesia bovis			*Babesia bigemina*			*Anaplasma centrale*		
Batch	Calf	Count x 10^6	Infect %	Calf	Count x 10^6	Infect %	Calf	Count x 10^6	Infect %
R058	9228	742	100	9085	204	92	9239	182	80
R059	9254	799	100	9094	260	92	9250	270	100
R061	9248	404	96	9240	182	96	9267	751	88
R062	9270	1204	96	9243	102	88	9269	681	96
R063	9277	1581	100	9258	133	96	9279	435	100
R064	9394	644	92	9242	128	92	9274	204	88
EA 43	8609	244	95	8611	89	90-100	8687	433	95
EA73-76	9258	435	76-92	9081	210	96-100	9252	183	88

Figure 1: Infectivity of experimental batches over time

The infectivity of the 3 parasites measured at 8, 24 and 36 hours is illustrated in the graphs below for the 4 experimental batches tested at Wandoan in 2009 (EA73 to EA76)






A. centrale
Percent infectivity
100
90
80
70
60
50
40
30
20
10
0
8 hr
24hr
36 hr
Vaccine shelf life
EA73
EA74
EA75
EA76

9.4 Appendix 4: Justification for Morven Trial 2009

9.4.1 Background

Important results from previous trials done in recent years were collated in order to provide a summary of results to help determine and justify the trial groups for a co-operator trial near Morven in September 2009 (9.5 Appendix 5, this report). This was the final trial prior to the conclusion of the MLA AHW132 project.

9.4.2 Chilled tick fever vaccine

The intention was to clarify the shelf-life with the view to extending the shelf-life beyond 4 days; to clarify the efficacy margin of the current dose; and to examine the need for the "die-off" factor used to increase the theoretical dose of parasites in stored blood (Day 0 and 1 = 1; Day 2 = 1.5; Day 3 = 3; Day 4 = 4.5; Day 5 = 6)

Babesia bovis

The indications are that *B. bovis* is a robust parasite which is highly infective even after 7 days and has a substantial efficacy margin in standard chilled diluent.

Tick Fever Trial 2007 (5 animal groups)

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	0	10	100%	Standard	
0	0	1	100%		
0	0	0.1	100%		
0	0	0.01	100%		
0	0	0.001	80%		
0	0	0.0001	60%		

Conclusion: Vaccine made from Day 0 blood (that is, on the day of collection) and used immediately was fully infective at 1/100th the standard vaccine dose.

Morven 2007

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	7	2	96%	Standard	
3	4	2	100%	Standard	
0	7	2	100%	AS24 alone	
0	7	2	100%	AS24 plus serum	
3	4	2	100%	AS24 alone	
3	4	2	100%	AS24 plus serum	
0	7	2	100%	Standard	Leukofiltered

Conclusion: Vaccine which had been made from Day 0 blood and stored for 7 days was fully infective; and vaccine which had been made from Day 3 blood and stored for 4 days was fully infective, even without inclusion of the standard "die-off" factor used with stored blood. *B. bovis* was also fully infective when used in diluent AS24 with or without serum; and when the blood was filtered to remove leucocytes.

Taroom 2008

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	4	2	100%	Standard	
0	4	1	100%	Standard	Validation of TM count
0	4	0.1	100%	Standard	
0	7	2	100%	Standard	
0	7	1	100%	Standard	Validation of TM count
0	7	0.1	96%	Standard	
0	14	1	96%	Standard	
0	14	0.1	100%	Standard	
4	4	9	100%	Standard	
4	4	4.5	100%	Standard	Validation of TM count
4	4	2	100%	Standard	
4	4	1	100%	Standard	
4	4	0.1	100%	Standard	

Conclusion: When made with fresh Day 0 blood, *B. bovis* was fully infective at 4 or 7 days, even at 1/10th the standard dose; when vaccine was made at the current limit of blood storage (4 days), *B. bovis* was fully infective at 1/45th the standard dose (standard vaccine made with Day 4 blood includes 4.5 times the number of parasites than when made with fresh blood, to account for "die-off")

Therefore the chilled *B. bovis* vaccine trial done at Morven was as follows:

Morven *B. bovis* 2009

Group	Blood storage	Vaccine storage	Count adj. factor	Die off factor	Titration dose	Final Dose (x10^7)
1	1	7	1	1	1	1
2	1	7	1	1	0.1	0.1
3	1	7	1	1	0.01	0.01
4	4	4	1	1	1	1
5	4	4	1	1	0.1	0.1
6	4	4	1	1	0.01	0.01
7	4	7	1	1	1	1
8	4	7	1	1	0.1	0.1
9	4	7	1	1	0.01	0.01

This trial aimed to:

- test the limits of infectivity of the standard dose of vaccine at 4 and 7 days made with Day 1 and Day 4 blood
- assess the impact of not including a "die-off" factor for vaccine made from Day 4 blood
- check efficacy margin at 1/10th and 1/100th dose
- give an indication of the possibility of extending the vaccine shelf-life beyond 4 days

Anaplasma centrale

The following trial results were considered in the design of the Morven 2009 trial.

St George 2005

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	4	4	100%	Standard	
0	4	4	94%	AS24	
0	4	4	100%	EAS76	
0	4	4	100%	SAGM-N	
0	7	4	95%	Standard	
0	7	4	22%	AS24	
0	7	4	32%	EAS76	
0	7	4	39%	SAGM-N	

Conclusion: Vaccine made with fresh (Day 0) blood was fully infective at 4 days (even when using serum-free diluents) and at 7 days with standard diluent (which contains 10% serum)

Tick Fever Trial 2007 (5 animal)

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	4	100	100%	Standard	
0	4	10	100%		
0	4	1	100%		
0	4	0.1	60%		
0	4	0.01	60%		
0	4	0.001	0%		

Conclusion: Vaccine made with fresh (Day 0) blood was fully infective at 4 days, down to ¼ the current dose, but not at 1/40th the current dose. The dose efficacy margin is small.

Morven 2007

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	7	4	96%	Standard	Trivalent
1	4	4	100%	Standard	Trivalent
3	4	4	100%	Standard	Trivalent
0	7	4	9%	AS24 alone	Trivalent
0	7	4	96%	AS24 plus serum	Trivalent
3	4	4	4%	AS24 alone	Trivalent
3	4	4	96%	AS24 plus serum	Trivalent
0	7	4	88%	standard	Leucofiltered

Conclusion: *A. centrale* vaccine may retain satisfactory infectivity for 7 days when made with fresh (Day 0) blood; or for 4 days when made with blood stored for 3 days. It does however require serum in the diluent to maintain infectivity.

Taroom 2008

Blood storage	Vaccine storage	Count x 10^7	Result	Diluent	Comments
0	4	4	100%	Standard	
0	4	1	100%	Standard	
0	4	2	100%	Standard	
0	7	4	100%	Standard	
0	7	1	36%	Standard	
0	7	2	68%	Standard	
0	14	1	4%	Standard	
0	14	2	0%	Standard	
1	4	4	100%	Standard	
1	4	2	100%	Standard	
1	4	1	100%	Standard	
3	4	1	100%	Standard	
3	4	3	88%	Standard	
3	4	6	100%	Standard	
3	4	12	100%	Standard	

Conclusion: *A. centrale* vaccine was fully infective at 4 days, whether vaccine was made with Day 0, Day 1 or Day 3 blood; it remained infective at ¼ the parasite dose at 4 days, but there was no efficacy margin at Day 7 – infectivity was reduced at ½ the standard dose.

Therefore the *A. centrale* trial done at Morven in 2009 was as follows:

Grp	Blood storage	Vaccine storage	Count adjustment factor	Die off factor	Titration dose	Final Dose (x10^7)	Diluent
1	1	4	4	1	4	4	Standard
2	1	4	1	1	1	1	Standard
3	1	4	1	1	0.1	0.1	Standard
4	1	7	1	1	1	1	Standard
5	1	7	1	1	1	1	20% serum
6	3	4	1	1	4	4	Standard
7	3	4	1	1	1	1	Standard
8	3	4	1	1	0.1	0.1	Standard
9	3	7	1	1	1	1	Standard
10	3	7	1	1	1	1	20% serum

The trial aimed to:

- clarify the dose of *A. centrale* needed for infectivity at 4 days and beyond
- clarify the efficacy margin at the current limit of blood and vaccine storage
- clarify if extra serum would help extend the shelf-life to 7 days in vaccine made from Day 1 or stored Day 3 blood (Groups 4-5 and 9-10)
- see if vaccine might be made from Day 3 blood, thereby allowing a once per week *A. centrale* blood collection (as for the *Babesia* spp parasites) rather than the current *A. centrale* twice weekly collection

9.4.3 Deglycerolised vaccine

The aim was to clarify the infectivity of deglycerolised and reconstituted frozen vaccine concentrate issued as a chilled product; to clarify the dose required and determine the likelihood that such vaccine would have an equivalent shelf-life of the current chilled vaccine.

Babesia bovis

The aim was to clarify the dose required for infectivity at 4 days, and see if it lasts to 7 days; this was to determine an infective dose and efficacy margin and then calculate how many doses we can get from a pack. This would allow us to consider further what role this procedure has at TFC.

Deglycerolisation is time consuming and we need to know how much time and how many people would be needed if this production system was adopted.

Morven 2008 and Wandoan 2009 dose comparisons – assuming that the dose was calculated by multiplying the original count by the ratio of the RBC count in the deglycerolised stock to the original donor calf RBC at collection; and that both infected and uninfected RBCs are lost at the same rate during the freeze, thaw and deglycerolisation processes.

Batch	Calf	Parasite count x 10^6/ml	Vaccine	Storage days	Dose x 10^7	Result
Morven 2008						
EA 53	8609	244	Triv	3	1.1	95%
				4		85%
				7		78%
			Dil 0.1	3	0.1	58%
			Dil 0.01	3	0.01	25%
EA 57	8970	326	Mono	3	6.3	100%
				4		95%
				6		100%
EA 56-1	8970	326	Mono	3	4.9	100%
				4		100%
				7		100%
			Dil 0.1	3	0.5	58%
			Dil 0.01	3	0.05	25%
				4	4.9	95%
				4	4.9	100%
EA 56-2	8970	326		4	4.6	95%
				4		100%
				4	0.5	85%
				4	0.05	42%
Wandoan 2009						
EA 72-1	9262	993	Mono	7	22.4	100%
				7 #	22.4	90%
				7	1	65%
			Dil 0.1	7	2.3	69%
			Dil 0.01	7	0.2	0%
EA 72-2	9262	993	Mono	3	24.4	100%
				3	1.1	68%
			Dil 0.1	3	2.5	85%
			Dil 0.01	3	0.2	50%
			Triv	3	24.4	100%
				4	24.4	94%
				4	1.1	67%
			Dil 0.1	4	2.5	70%
			Dil 0.01	4	0.2	50%
				3 #	24.4	90%

Batch	Calf	Parasite count x 10^6/ml	Vaccine	Storage days	Dose x 10^7	Result
			Triv	4	24.4	100%
				4 #	24.4	83%
			Triv	7	24.4	89%

Deglycerolised vaccine stock stored 1 day prior to reconstitution

It seems from the above fairly clear that, even with the inaccuracies in dose estimation, that there is a very strong dose-dependent relationship – infectivity was good at a dose greater than about 5 x 10^7 parasites at Morven 2008 or greater than 10 x 10^7 at Wandoan 2009; and fell away once the dose was around 1 x10^7. However, infectivity was fairly constant across 3, 4 or 7 days suggesting that the viable parasites present at the start will survive (except at very low dilutions). Storage of the deglycerolised vaccine concentrate for 1 day prior to reconstitution reduced infectivity slightly.

Therefore the proposed trial deglycerolised *B. bovis* trial for Morven in 2009 was:

Group	Parasite dose			Serum concentration		Vaccine storage days	
	1 x 10^6	1 x 10^7	1 x 10^8	10% serum	20% serum	4d	7d
B10	x			x		x	
B11		x		x		x	
B12		x		x			x
B13			x	x		x	
B14			x	x			x
B15	x				x	x	
B16		x			x	x	
B17	x			x			x
B18		x			x		x

This will clarify the dose required to achieve satisfactory infectivity and efficacy margin; and also indicate if extra serum at the lower dose rates helps support the parasites and ensure improved infectivity

Anaplasma centrale

Deglycerolised *A. centrale* has never proved to be fully infective at 3 days post-reconstitution. The aim is to see if we can get *A. centrale* to survive and be fully infective at both 3 and 4 days. Fresh vaccine work suggests that infectivity of *A. centrale* falls away quite quickly below the standard vaccine dose; and when vaccine is used beyond the 4 day shelf-life.

Wandoan 2009 results

1. *Serum content*

Groups to compare	20% serum	10% serum
A1 vs A2	69%	31%
A3 vs A4	80%	10%
A8 vs A10	53%	7.7%
A6 vs A7	66%	44%
A11 vs A13	20%	30%

Conclusion: The results fairly strongly suggest that infectivity is improved by higher serum content.

2. *0.5M vs 0.3M sorbitol wash*

Groups to compare	0.5 sorbitol	0.3 sorbitol
A8 vs A9	53%	8%
A11 vs A12	20%	10%

Conclusion: 0.5 M sorbitol favoured

3. *Leucodepleted or not*

Groups to compare	Leucodepleted	Not
A8 vs A11	53%	20%
A9 vs A12	8%	10%
A10 vs A13	8%	30%

Conclusion: Despite inconclusive results, leucodepleted vaccine concentrate is easier to process.

1. *Pre-freeze cryo-expression vs not cryo-expressed or post-thaw expressed*

Groups to compare	Pre-freeze cryo-expressed	Not expressed	Post thaw cryo-expressed
A3 vs A1		69%	80%
A4 vs A2		32%	15%
A6 vs A5		55%	66%
A8 vs A3	53%		80%
A11 vs A6	20%		55%

Conclusion: There may be some advantage to expressing the cryopreservative after thawing and prior to processing; but this is not completely clear.

We know from chilled work that *A. centrale* won't last without serum and needs >1 x 10^7 for infectivity. So the aim here was to ensure that we give plenty of serum (up to 30%) and that we have plenty of parasites, so check dose required at standard dose or higher (1 x 10^7 and 1 x 10^8) – this probably should be 4 x 10^7 and 4 x 10^8 to mimic current vaccine with count adjustment factor.

The proposed trial deglycerolised *A. centrale* trial for Morven in 2009 was:

Group	Parasite	Parasite dose		Serum concentration		Vaccine storage	
		4 x 10^7	4 x 10^8	20% serum	30% serum	3d	4d
11	*A. centrale*	x		x		x	
12	*A. centrale*		x	x		x	
13	*A. centrale*	x		x			x
14	*A. centrale*		x	x			x
15	*A. centrale*	x			x	x	
16	*A. centrale*		x		x	x	
17	*A. marginale*	x		x		x	
18	*A. marginale*		x	x		x	
19	*A. marginale*	x			x	x	
20	*A. marginale*		x		x	x	

This trial will test:

- The infectivity of a dose equivalent to the standard fresh vaccine dose and a dose 10 times that
- The effect on infectivity of extra serum (20% and 30% serum) compared to standard diluent with 10% serum, using a production process for *A. centrale* bags which involves leucodepletion, concentrated 1:1 with post-thaw expression and automated deglycerolisation

This trial will also give an indication of whether *A. marginale* is infective following the freeze, thaw and deglycerolisation process. *A. marginale* Dawn batch M001 does not have a recorded parasitaemia, and as this batch will be made from tubes using a manual deglycerolisation protocol with no RBC count, only estimates of dose will be available.

9.5 Appendix 5: Chilled vaccine and deglycerolised reconstituted frozen vaccine: validation of infectivity and dose – Morven 2009

9.5.1 Background

This was the final animal trial as part of MLA project AHW 132. Previous animal trials on co-operator properties showed that:

- the infectivity of deglycerolised *B. bovis* RBCs was very high using an automated process, albeit at a much higher theoretical dose than that used in chilled vaccine
- the infectivity of deglycerolised *A. centrale* RBCs did not persist more than 2 days after reconstitution, even with increased concentration of serum in the diluent
- *B. bovis* remained highly infective up to 14 days in chilled vaccine, even at much reduced doses
- *A. centrale* in chilled vaccine was infective up to 7 days, but with little efficacy margin at reduced doses, even at 4 days
- Results for *B. bigemina* in recent trials were inconsistent with our knowledge of the infectivity of this parasite built up over some years, such that further clarification of the serological test was warranted in smaller trials to be conducted at TFC before we attempt doing *B. bigemina* trials to cooperator properties again.

Furthermore, consideration is being given to developing a “mild” and protective field isolate of *A. marginale* (“Dawn” strain) as the vaccine strain to replace *A. centrale* in the vaccine.

The aim of this trial was to look further at the following:

Deglycerolised *B. bovis* - duration of infectivity after deglycerolisation and reconstitution, clarification of the theoretical dose, and logistics and capability of producing chilled vaccine from a bulk frozen vaccine concentrate.
Deglycerolised *A. centrale* – assessment of an automated deglycerolisation process, clarification of the theoretical dose, and assessment of increased serum concentration as a means of extending the shelf-life to 3 and/or 4 days and improving the efficacy margin.
Deglycerolised *A. marginale* (Dawn) – the effect of manual deglycerolisation in a small pilot trial to see if it might be better suited to this process than *A. centrale*
Chilled *B. bovis* and *A. centrale* vaccine – clarification of the infective dose required at the limits of blood and vaccine storage; and assessment of the feasibility of extending the shelf-life of the chilled vaccine.

9.5.2 Materials and Methods

Experimental cattle

Access to approximately 450 weaned cattle was secured on a cattle tick-free co-operator property near Morven; these were divided into groups as follows:

Chilled *B. bovis* – 9 groups of 25 head
Deglycerolised *B. bovis* – 9 groups of 25 head
Chilled *A. centrale* – 10 groups of 23 head
Deglycerolised *A. centrale* – 6 groups of 22 head
Deglycerolised *A. marginale* – 4 groups of 22 head

The details of the various groups and treatments are shown in Tables 1, 2 and 3. All vaccines were prepared as monovalent vaccines. The cattle were inoculated, and a sample bled and tested for antibodies to tick fever in August 2009; and then were bled and tested again in November 2009 (about 70 days later) to assess the rate of seroconversion in the different groups. Individual animals were inoculated on two occasions using monovalent vaccines made

from different organisms. The presence of antibodies to the vaccine organisms was taken as a definitive indication of vaccine potency.

The trial was conducted with the approval of an Animal Ethics Committee and the APVMA. The co-operator monitored the cattle for possible vaccine reactions and reported that none were detected.

Experimental design

Blood containing the different organisms was collected from vaccine donor calves infected in accordance with routine vaccine production procedures. Chilled vaccine was made from blood stored for 1 or 4 days (*B. bovis*) or 1 or 3 days (*A. centrale*). Deglycerolised vaccine was made from fresh blood which was processed, glycerolised and frozen in bags (*B. bovis* and *A. centrale*) or tubes (*A. marginale*) as outlined below. These frozen concentrates were stored in liquid nitrogen, then thawed and deglycerolised as outlined below. The *B. bovis* and *A. centrale* chilled vaccines were mainly reconstituted in standard chilled vaccine diluent containing 10% serum while the *A. centrale* and *A. marginale* vaccines were reconstituted in standard diluent with 20% or 30% serum. Prior to inoculation, vaccines were stored for periods of 3, 4 and 7 days after reconstitution. All vaccines were also administered at varying doses in order to evaluate the efficacy margin. Calculations of the dose rates for bulk frozen vaccine concentrate were based on the RBC count in the deglycerolised vaccine concentrate compared to the RBC count in the original vaccine donor blood.

Glycerolisation Methods

Glycerolised frozen *B. bovis* vaccine (batch EA79) was prepared following the standard protocol for the glycerolisation described previously in Appendix MS4-4.

For *A. centrale* glycerolised frozen vaccine, batch EA77 (concentrated blood, stored 1:1 with glycerol (*A.centrale* 4M GPBSG conc., 1:1)), freshly collected infected blood was leucofiltered then concentrated by centrifugation at 2429g for 15 minutes. The supernatant was aspirated and discarded. An equal quantity of 4M glycerol in PBS with glucose (GPBSG) pre-warmed to 37°C was added aseptically, and the mixture was incubated at 37°C in a water bath for 30 minutes. Final PCV of the glycerolised iRBC mix was about 35%. The blood was then transferred into 200mL cryocyte bags, frozen for 15 minutes each side in the gaseous phase of liquid nitrogen before submersion and storage in liquid nitrogen.

A. marginale monovalent frozen vaccine (batch M001, prepared in 2001) was prepared following traditional methods of monovalent frozen vaccine. An equal quantity of 3M glycerol in PBS with glucose (GPBSG), pre-warmed to 37°C, was added aseptically to whole infected blood. The blood was then incubated in a 37°C water bath for 30 minutes before freezing in the gaseous phase of liquid nitrogen for 15 minutes prior to submersion and storage in liquid nitrogen. No parasite count was recorded for this batch.

These glycerolisation protocols are summarised in Table 1.

Table 1: Glycerolisation procedure summary

Vaccine batch	Organism	CPA	Leuco-depletion via filtration	Centrifugation/ concentration of blood prior to addition of glycerol	Centrifugation/ expression of excess CPA after addition of glycerol and prior to freezing	Final PCV prior to freezing	Storage in Iwaki cryotubes or cryocyte bags
EA77	*A. centrale*	4M glycerol	Yes	Yes	No	43	200mL bags
EA79	*B. bovis*	4M glycerol	Yes	No	Yes	82	200mL bags
M001	*A. marginale*	3M glycerol	No	No	No	Approx 14	5mL tubes

Deglycerolisation Methods

Vaccine was deglycerolised following the standard method for tubes and bags as outlined previously (Appendices MS4-4 and MS4-5), except for variations shown in Table 2 for vaccine in bags and in Table 3 for vaccine in tubes. For the automated deglycerolisation in bags, the exposure buffer for each bag was 2M GPBSG + 1M sorbitol at 1x volume, with 2 dilutions using 0.5M sorbitol in PBSG (both at 2x volume), and 4 washes using 0.3M sorbitol in PBSG (volumes of 60mL, 70mL, 100mL and 150mL).

Table 2: Deglycerolisation procedure summary – vaccine stored in bags, automated deglycerolisation using Haemonetics ACP215 machine

Vaccine batch	Bag	Haemonetics protocol	Animal group no	Vaccine diluent	Estimated parasites per dose	Vaccine storage (days)
EA79 *B. bovis*	1	Protocol A (16.667min equil, continuous addition of dilutions, shaker, 4 washes)	10	Standard chilled	1×10^6	4
			11	Standard chilled	1×10^7	4
			12	Standard chilled	1×10^7	7
			13	Standard chilled	1×10^8	4
			14	Standard chilled	1×10^8	7
			15	Standard chilled plus 20% serum	1×10^6	4
			16	Standard chilled plus 20% serum	1×10^7	4
			17	Standard chilled	1×10^6	7
			18	Standard chilled plus 20% serum	1×10^7	7
EA77 *A. cent*	1	Protocol A (16.667min equil, continuous addition of dilutions, shaker, 4 washes)	11	Standard chilled plus 20% serum	4×10^7	3
			12	Standard chilled plus 20% serum	4×10^8	3
			13	Standard chilled plus 20% serum	4×10^7	4
			14	Standard chilled plus 20% serum	4×10^8	4
			15	Standard chilled plus 30% serum	4×10^7	3
			16	Standard chilled plus 30% serum	4×10^8	3

Table 3: Deglycerolisation procedure summary – vaccine stored in tubes, manual deglycerolisation

Animal group no	Vaccine batch	Exposure buffer	Equil time (min)	Centrifuge	Wash 1	Wash 2	Vaccine diluent	Dilution †	Vaccine storage (days)
17	M001 (*A marg*)	1.5M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	1/100	3
18	M001 (*A marg*)	1.5M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 20% serum	1/10	3
19	M001 (*A marg*)	1.5M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 30% serum	1/100	3
20	M001 (*A marg*)	1.5M GPBSG + 1M sorbitol	90	Std (3010g)	0.5M sorb in PBSG (1/10)	0.5M sorb in PBSG (1/10)	Standard chilled plus 30% serum	1/10	3

Note: † The equivalent of 1mL of thawed vaccine was diluted to 10mL (or 100mL).

9.5.3 Results and Discussion: Chilled *Babesia bovis* vaccine

Results are shown in Table 4. Standard vaccine at the limit of the current registration shelf-life (4 days blood storage and 4 days vaccine storage) retained infectivity at 1/10th the standard dose without any allowance for "die-off" in stored blood. This equates to 1/45th the number of parasites that would normally be included when vaccine is made from blood stored for 4 days. When this same vaccine was used at 7 days after manufacture, infectivity was declining at the standard dose (without die-off allowance); that is, the efficacy margin was substantially reduced. Vaccine made with Day 1 blood and stored for 7 days, also showed good infectivity at the standard dose, but efficacy was slightly reduced at 1/10th the standard dose.

These results indicate considerable opportunity to extend the shelf-life of the *B. bovis* component in the current standard chilled vaccine. The shelf-life of *B. bovis* in vaccine would, however, depend somewhat on what is deemed an appropriate efficacy margin; and also what the likely limits of blood storage would be if the number of days of manufacture per week was reduced in a new system of vaccine production where vaccine was made less frequently but has a shelf-life beyond 4 days. Further trials are needed to clarify the infectivity and efficacy margin of *B. bovis* with various permutations of blood storage (from 1-4 days) and vaccine storage (from 4-7 days).

9.5.4 Results and Discussion: Chilled *Anaplasma centrale* vaccine

The results for *A. centrale* are summarised in Table 5. Vaccine tested to the limits of current registration (vaccine made with Day 1 blood and stored for 4 days) was infective at ¼ the standard dose, but infectivity was markedly reduced at 1/40th standard dose. The same vaccine stored for 7 days showed poor infectivity at ¼ the standard dose, even with the addition of extra serum to the diluent. Despite good infectivity in an earlier trial (Appendix MS5-3), vaccine made from Day 3 blood and stored for 4 days showed poor infectivity in this trial, even at the standard dose with no allowance for die-off in the stored blood. When blood was stored for 3 days (to mimic one *A. centrale* blood collection per week), vaccine had poor infectivity at 4 and 7 days.

A. centrale vaccine appears to have a relatively limited efficacy margin (certainly less than 10-fold); and limited survival beyond 4 days. Further trials will aim to clarify the dose above 1 x 10^7 needed for satisfactory efficacy margins and shelf-life at 5-6 days. *A. centrale* will be the limiting organism in any attempt to extend the shelf-life of the chilled trivalent vaccine beyond 4 days.

9.5.5 Results and discussion: Deglycerolised *Babesia bovis* vaccine

Results are shown in Table 6. The only vaccine groups which were fully infective, both at 4 and 7 days after vaccine manufacture, contained a theoretical dose of 1 x 10^8 parasites. At doses of 1 x 10^7 parasites, 20% serum in the diluent seemed to improve infectivity slightly. Vaccine infectivity was closely related to dose in all similar groups.

Attempts to use a direct parasite count when calculating the volume of deglycerolised vaccine concentrate to be included in the vaccine met with little success because of the difficulties in making suitable slides for examination. Parasite inclusion rates were therefore based on theoretical calculations of the RBC count of the deglycerolised vaccine concentrate stock compared to the RBC and parasite count in the fresh blood from the donor calf, assuming that both infected and uninfected RBCs are lost at the same rate in the freeze, thaw and deglycerolisation processes. The calculated dose is therefore theoretical only. The results indicate that a calculated dose 10 times the standard chilled vaccine *B. bovis* parasite dose must be included in deglycerolised vaccine to achieve satisfactory infectivity, albeit with a less than 10-fold efficacy margin. At this dose rate and with the parasite count in the original donor blood, one bulk frozen bag provided only about 2400 doses; much less than that required to meet the daily *B. bovis* demand in all but a few weeks in the summer months.

9.5.6 Results and discussion: Deglycerolised *Anaplasma centrale* and *Anaplasma marginale* vaccine

The results are summarised in Table 7.

A. centrale infectivity was highly dose dependent; all groups with a theoretical dose of 1 x 10^8 parasites achieved better infectivity than their counterpart with 1 x 10^7 parasites. The only group with satisfactory infectivity at 3 days after vaccine manufacture contained 30% serum (3 x standard concentration) in the diluent. No group was infective to 4 days.

Dawn *A. marginale* showed very good infectivity at 3 days. There was some demonstrated advantage with the addition of 30% serum to the diluent in the groups with a putative dose of 1 x 10^7 parasites. This was just a pilot trial using a manual deglycerolisation process, and given the original parasitaemia of the vaccine concentrate was not known (manufactured in 2001) the dose estimates are at best "guestimates". Results were, however, sufficiently promising to warrant further investigation of the shelf-life of Dawn *A. marginale*, even in chilled vaccine. This work will be continued after the end of this project as previous trials have shown that this strain is both mild and more protective than *A. centrale* against field strains of *A. marginale.*

Table 4: Chilled *Babesia bovis* vaccine infectivity

Group Name	No. of animals	Blood storage	Vaccine storage	Fudge factor	Die off factor	Titration dose	Final Dose (x107)	Group in words	Comment	DPF%
B1	23	4	7	1	1	1	1	see If long storage blood is still infective (without compensation for die off) - another permutation of 0/14 scenario	max blood storage plus 7 day vaccine shelf life	100%
B2	24	4	7	1	1	0.1	0.1	see if above vaccine has at least 10x efficacy margin	efficacy margin for Group B1	71%
B3	25	4	7	1	1	0.01	0.01	see if above vaccine has at least 100x efficacy margin	efficacy margin for Group B1	24%
B4	24	4	4	1	1	1	1	see if max likely storage of blood WITHOUT compensation for die off infective when 4d stored vaccine used with current starting dose	repeat of Taroom 08 (Group 12) - validate current vaccine w/o fudge or die-off factors	100%
B5	25	4	4	1	1	0.1	0.1	see if above vaccine has at least 10x efficacy margin	repeat of Taroom 08 (Group 13)	96%
B6	25	4	4	1	1	0.01	0.01	see if above vaccine has at least 100x efficacy margin	to help determine efficacy margin of current vaccine count after 4+4 days	68%
B7	23	1	7	1	1	1	1	see if Day 1 blood and stored vaccine (d7) infective using current starting dose	almost same as Morven07, also Taroom 08 (Group 5) except blood storage day 1	100%
B8	24	1	7	1	1	0.1	0.1	see if above vaccine has at least 10x efficacy margin	almost same as Taroom 08 (Group 6)	92%
B9	25	1	7	1	1	0.01	0.01	see if above vaccine has at least 100x efficacy margin	to help determine efficacy margin after 7 days	56%

Table 5: Chilled *Anaplasma centrale* vaccine infectivity

Group Name	No. of animals	Blood storage	Vaccine storage	Count adjustment factor	Die off factor	Titration dose	Final Dose ($x10^7$)	Group in words	Comment	DPF %
A1	23	1	4	4	1	4	4	standard vaccine	repeat of titration trial	96%
A2	23	1	4	1	1	1	1	standard vaccine - no fudge factor	100% at Taroom 08	96%
A3	22	1	4	1	1	0.1	0.1	0.1 dose titration of Group 2	test limits of titration	48%
A4	23	1	7	1	1	1	1	standard vaccine - no fudge factor - at 7 days	0/7 was 38% at Taroom 08 - to check efficacy margin of 7 day *A. centrale* vaccine	41%
A5	23	1	7	1	1	1	1	standard vaccine - no fudge factor - at 7 days, with 20% serum	with 20% serum diluent	24%
A6	23	3	4	1	1	4	4	3 day blood, 4 day vaccine, no allowance for die-off	88% at Taroom 08	77%
A7	23	3	4	1	1	1	1	3 day blood, 4 day vaccine, no fudge, no die-off	100% at Taroom 08	57%
A8	23	3	4	1	1	0.1	0.1	0.1 dose titration of Group 15	test limits of titration	5%
A9	23	3	7	1	1	1	1	3 day blood, 7 day vaccine, no fudge, no die-off	test limits of titration	30%
A10	22	3	7	1	1	1	1	3 day blood, 7 day vaccine, no fudge, no die-off, with 20% serum	test limits of titration	38%

Table 6: Deglycerolised *Babesia bovis* vaccine infectivity

Group Name	No. of animals	Batch	Post-thaw processing		Parasites per dose			Diluent type		Vaccine storage		DPF %
			No. bags deglyced	Deglyc type Std ACP	1×10^6	1×10^7	1×10^8	10% serum	20% serum	4d	7d	
B10	25	EA79			x			x		x		36%
B11	24	EA79	1	x		X		x		x		63%
B12	23	EA79	1	x		X		x			x	35%
B13	24	EA79	1	x			x	x		x		100%
B14	25	EA79	1	x			x	x			x	96%
B15	25	EA79	1	x	x				x	x		24%
B16	23	EA79	1	x		X			x	x		78%
B17	23	EA79	1	x	x			x			x	13%
B18	24	EA79	1	x		X			x		x	54%

Table 7: Deglycerolised *Anaplasma centrale and Anaplasma marginale* vaccine infectivity

Group Name	No. of animals	Batch	Post-thaw processing				Parasites per dose		Diluent		Days Storage		
			Deglyc washes		Automatic deglyc Haemonetics								
			Wash 1 Solution Type	Wash 2 Solution Type	16.67min equil	Std ACP	4×10^7	4×10^8	20% serum	30% serum	3d	4d	DPF %
A11	22	EA77	1	1	x	x	x		x		x		43%
A12	22	EA77	1	1	x	x		x	x		x		82%
A13	22	EA77	1	1	x	x	x		x			x	23%
A14	22	EA77	1	1	x	x		x	x			x	64%
A15	21	EA77	1	1	x	x	x			x	x		55%
A16	22	EA77	1	1	x	x		x		x	x		96%
A17	22	M001	Manual				x		x		x		81%
A18	22	M001	Manual					x	x		x		100%
A19	22	M001	Manual				x			x	x		96%
A20	22	M001	Manual					x		x	x		100%

9.6 Appendix 6: SOP VPL25 Combavac formulation, dispensing and freezing

See pdf file attached

10 Abbreviations and acronyms

ABCRC	Australian Beef and Cattle Research Committee
AMRC	Australian Meat Research Committee
APVMA	Australian Pesticides and Veterinary Medicines Authority
cELISA	Competitive Enzyme Linked Immunosorbent Assay
CPA	Cryoprotective Agent
Dil.	Diluent
DMSO	Dimethyl Sulphoxide
DPI	Department of Primary Industries
DPI&F	Department of Primary Industries & Fisheries
DEEDI	Department of Employment, Economic Development and Innovation
DPF	Disease Prevalence Factor
ELISA	Enzyme Linked Immunosorbent Assay
GPBSG	Glycerol in phosphate buffered saline with glucose
HCT	Haematocrit
iRBC	Infected Red Blood Cell/s
MLA	Meat and Livestock Authority
MoAb	Monoclonal antibodies
Mono.	Monovalent
NR	Non-reactive
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PI	Percentage inhibition
R	Reactive
RBC	Red Blood Cell/s
SD	Standard Deviation
Std	Standard
Sorb.	Sorbitol
SOP	Standard Operating Procedure
Susp	Suspect
TFC	Tick Fever Centre
TM	Traditional Counting Method
Triv.	Trivalent
uRBC	Uninfected red blood cell/s