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Final report

Improved protection of cattle against anaplasmosis in tick-infested areas of Australia

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Abstract

Trial steers were inoculated with three different batches of both experimental chilled and frozen trivalent vaccines consisting of *Babesia bovis*, *Babesia bigemina* and Dawn strain *Anaplasma marginale*. Safety, virulence, infectivity and efficacy were compared with the current registered chilled and frozen trivalent tick fever vaccine containing both of the *Babesia* spp and *Anaplasma centrale*. Whilst there was no difference in safety and virulence of the experimental vaccines, infectivity of the A. *marginale* was poor in some of the vaccines, and unexplained lack of virulence in the challenge strain makes interpretation of some aspects of the trial difficult. Dawn strain A. *marginale* was also shown again to be poorly tick transmissible. It will not be possible to register Dawn strain A. *marginale* as a vaccine strain without further evaluation.

Executive summary

A. marginale is endemic in tropical and subtropical areas of the world where it causes significant economic loss to the cattle industries. Anaplasmosis accounted for about 14% of the confirmed tick fever outbreaks in Queensland in the period 1990 to 2009.

Anaplasma centrale, the current *Anaplasma spp* strain in trivalent tick fever vaccine, imparts partial and variable immunity against challenge with Australian isolates of *A. marginale*. *A. centrale* is not transmitted by *Rhipicephalus (Boophilus) microplus* or other ticks present in Australia. There are occasional outbreaks recorded of anaplasmosis in herds where serological evidence indicated that *A. centrale* vaccine had provoked an immune response, but the protection provided was inadequate. Despite this, field evidence suggests that it provides adequate control on most properties against the effects of *A. marginale* in Australia. *A. centrale* is not completely benign and is known to cause anaemia, and the principal reason for the very short four day shelf life of the chilled tick fever vaccine is the rapid loss of potency of the *A. centrale* strain. This limits the availability of the chilled vaccine to users under some circumstances.

The identification of naturally occurring *A. marginale* isolates that produce mild infections in cattle and protect against virulent challenge has eluded most efforts. The original assessment in 2003 however, of one Australian isolate of *A. marginale* (Dawn strain) indicated it was as least as mild as *A. centrale* and provided almost total protection against challenge with heterologous Australian field isolates of *A. marginale*. This study also indicated it was poorly tick transmissible, as two attempts to artificially transmit this Dawn isolate in pen trials using *R. (B.) microplus* were unsuccessful. In field trials conducted in 2010, there was every indication that Dawn strain *A. marginale* was very infective and the infectivity persisted well in the vaccine post-manufacture.

Dawn *A. marginale* is currently available under Australian Pesticides and Veterinary Medicines Authority (APVMA) Minor Use Permit as a monovalent frozen vaccine. It has been used successfully and without adverse reactions since 2004 on two properties where *A. centrale* vaccination had failed to prevent anaplasmosis.

Given the mild nature of Dawn strain *A. marginale*, the advantages it offered in terms of better protection of cattle against anaplasmosis and the potential to extend the shelf life, the intention of this project was to further evaluate this strain in line with APVMA requirements for registration of immunobiologicals (Module 8.3 – Safety and Efficacy); to clarify tick transmissibility; and to find a molecular marker that would allow differentiation of Dawn strain *A. marginale* in the vaccine from *A. marginale* field isolates. The aim was to replace *A. centrale* with Dawn strain *A. marginale* in the registered chilled and frozen tick fever vaccines.

Initially a pilot trial was conducted to clarify the dose of Dawn *A. marginale* to be used in the main vaccine trials. Thereafter target animal safety and efficacy studies were conducted in accordance with APVMA guidelines. Evaluation of efficacy included both infectivity (that is, a patent *Anaplasma spp* infection was established and an immune response developed following vaccination) and the ability to withstand challenge from a virulent *A. marginale* isolate. Standard chilled trivalent and frozen Combavac 3in1 tick fever vaccines containing *A. centrale*, and previously manufactured monovalent Dawn strain *A. marginale* frozen batches M001 and M004, were used as controls for comparison. Three batches each of Dawn strain *A. marginale* frozen and chilled vaccines were tested in a trivalent vaccine which included the standard *Babesia bovis* and *Babesia bigemina* vaccine strains. The vaccines were administered on different dates determined by passage number of the *A. marginale* vaccine donor calf and date of manufacture (determined by blood and vaccine storage). Vaccination occurred in three subgroups: Groups 1–8 were used to assess safety, infectivity and

virulence; Groups 9–11 infectivity only and Groups 12– 17 infectivity and virulence. The reaction period for *Anaplasma spp* is expected 30 to 60 days after vaccination and intensive monitoring was conducted in that period.

The pilot trial confirmed that a dose of 1 x 10^7 organisms was sufficient to result in patent infection after vaccination. In the safety, infectivity, virulence and efficacy trials, there were no observable adverse systemic reactions in any animals and none required treatment in the immediate post-vaccination or *Babesia spp* reaction period. However, Dawn strain *A. marginale* infectivity across the vaccine groups was poor and variable (14%–100%) based on microscopic examination, polymerase chain reaction (PCR) and serology results. Assessment of virulence was based on red blood cell packed cell volume (PCV) depression, using only steers which were considered to have been infected with Dawn strain following vaccination. The PCV depression for standard dose vaccine groups containing either *A. marginale* or *A. centrale* ranged from about 16% to 27% (Groups 1–8), and no differences were attributable to either *A. marginale* or *A. centrale* in the vaccine. Poor infectivity in Groups 12–17 precluded any useful analysis of virulence in these groups. There is the possibility that infectivity declines with continuing passage; this would need further evaluation.

Evidence of infection and effect of challenge with virulent Gypsy Plains strain *A. marginale* (especially in the unvaccinated controls and *A. centrale* groups) was much less than expected from previous trial work. Only one of seven naïve control animals developed detectable parasitaemia and required treatment, although all control animals were shown to be infected by other means. This apparent lack of virulence of Gypsy Plains strain was not anticipated and made interpretation of results problematic. When considered with poor infectivity of Dawn strain *A. marginale* vaccines, these two factors conceal (in this trial at least) any differences that may exist in the level of protection imparted by Dawn *A. marginale* vaccine compared to *A centrale*.

The tick transmissibility trial confirmed that Dawn *A. marginale* is not readily transmitted by the cattle tick *R. (B.) microplus*, although more evidence would be required to exclude transmission completely. This is an important consideration, as a PCR assay has been developed which can distinguish Dawn strain *A. marginale* from other field isolates; and could potentially be used to identify vaccinated cattle.

Benign *Theileria spp* were identified in all steers in all trials. The subclinical anaemia induced by *Theileria spp* resulted in delays to the start of the trials. There is evidence in the literature that *Theileria spp* infection may affect the response to *A. marginale* infection, but the extent to which this affected these current trials is not clear.

There is some corroboration of a previous study where Dawn strain *A. marginale* proved to be as safe as, and no more virulent than *A. centrale*. Reasons for poor infectivity and the effect of *Theileria spp* infection on response to *A. marginale* infection need to be further investigated; and the challenge study needs to be repeated before consideration is given to registration of Dawn *A. marginale* as a vaccine strain.

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1. Background

1.1 Anaplasmosis in Australia

A. marginale is endemic in tropical and subtropical areas of the world where it causes significant economic loss to the cattle industries[1]. The organism was first implicated as the specific cause of anaplasmosis of cattle, and noted to be transmitted by cattle tick, by Theiler in South Africa in 1910. The disease is characterised by anaemia, jaundice and weight loss.

A. marginale was first identified in Australia by Legg in 1932[2]. However it is thought the tick fever complex (anaplasmosis and babesiosis) was introduced in the 1800s into northern Australia with cattle from Timor and perhaps Bali carrying the cattle tick *Rhipicephalus (Boophilus) microplus*; and was apparently endemic in the Darwin area by 1870. For many years it had a major effect on the cattle industry in northern Australia. The history of the introduction and efforts to control these diseases in Australia has been described by Angus (1998) [2].

Anaplasmosis accounted for about 14% of the confirmed tick fever outbreaks in Queensland in the period 1990 to 2009 (QLD DPI and DEEDI records). Clinical evidence suggests that *A. marginale* isolates in Australia do not differ significantly in either antigenicity or virulence[3] and genetic studies of local isolates suggest limited introduction with the tick vector *R. (B.) microplus* during the 19th century. In other countries, isolates of *A. marginale* differ more in antigenic composition, protection afforded against heterologous challenge, pathogenicity and tick transmissibility[4].

The identification of naturally occurring *A. marginale* isolates that produce mild infections in cattle and protect against virulent challenge has eluded most efforts. Local Australian strains of *A. marginale* proved too virulent to use as vaccines to protect against anaplasmosis in the field. Theiler in South Africa had noted the organism *Anaplasma centrale* provided a "high degree of resistance to infection with *A. marginale*" [2]. Thus, in 1934, *A. centrale* (Onderstepoort strain) was introduced into quarantine in Australia in two live carrier animals from South Africa; and since then has been used to immunise many millions of cattle in this country against anaplasmosis.

A. centrale only imparts partial and variable immunity against challenge with Australian isolates of *A. marginale*[3]. Despite this, field evidence suggests that it provides adequate control on most properties against the effects of *A. marginale* in Australia (TFC unpublished). There are however, outbreaks recorded of anaplasmosis in herds where there is serological evidence that *A. centrale* vaccine had provoked an immune response, but the protection provided was inadequate (TFC unpublished)[3]. On such properties where protection failure occurs after vaccination, losses are too persistent to ignore and pose a real problem for the producer and vaccine manufacturer.

Furthermore, *A. centrale* is not completely benign and is known to cause anaemia. In fact, the only reported and confirmed reactions where tick fever vaccine has caused clinical disease in recent years in Australia have been attributed to the *A. centrale* component of the vaccine, and not to either of the *Babesia spp* included in the vaccine (TFC unpublished). In one trial, significant reduction in weight gain was observed during development of immunity to *A. centrale* (TFC unpublished), which may be important for example in animals close to market weight.

The principal reason for the short four day shelf life of the chilled tick fever vaccine is the rapid loss of potency of the *A. centrale* strain. This limits the availability of the chilled vaccine to users under some circumstances.

A. centrale is not transmitted by *R. (B.) microplus* or other tick species present in Australia. This has proved a useful trait, as evidence of *A. centrale* infection in an animal therefore provides evidence of successful vaccination. A competitive ELISA to detect antibody to *A. centrale* is routinely used as part of investigations into vaccine efficacy and failures in the field[5].

1.2 Types of vaccines against anaplasmosis

Both live and killed vaccines against anaplasmosis are described[1]. Live *A. centrale* vaccine is the most widely used. The *A. centrale* organism is propagated in splenectomised calves; and the harvested infected red blood cells form the active basis of the vaccine. In Australia, these *A. centrale* infected red blood cells are mixed with red blood cells derived from calves infected with *Babesia bovis* and *Babesia bigemina*, plus diluent, to produce trivalent tick fever vaccine. The vaccine is distributed chilled with a four day shelf-life. With this type of vaccine comes the risk of unwanted spread of other disease agents and this must be countered with strict quarantine protocols. A single successful vaccination establishes persistent infection in the animal and is sufficient to provide immunity for life. A frozen form of the vaccine known as Combavac 3in1, which also contains *B. bovis*, *B. bigemina* and *A. centrale*, but must be stored and transported in liquid nitrogen, is available for larger and more remote properties where the logistics of shipping chilled vaccine is problematic; and also for export.

Whilst attenuated and killed vaccines have been tested experimentally and a killed vaccine was registered in the USA for some years prior to 2000, they have not provided as good protection as live vaccines, especially against different *A. marginale* genotypes, and require yearly boosters to maintain adequate immunity. Advances in molecular technology have not yet provided the answers to a reliable killed or attenuated vaccine which will provide broad cross protection against anaplasmosis.

1.3 Dawn strain *Anaplasma marginale*

In 1991, *A. marginale* infection was detected in a calf brought to Tick Fever Centre (TFC) from a cattle tick-free area of western Queensland, about one month after it was splenectomised[4]. The calf's dam (a 14-year-old cow known as Dawn) had also been born in the cattle tick-free area. The grand-dam however had come from a cattle tick infested property. The dam (Dawn) was also subsequently shown to be infected, but the grand-dam was not available for testing. It is therefore assumed (in the absence of evidence otherwise) that the calf's infection originated via the transplacental route over two generations.

Initial evaluation of Dawn strain *A. marginale* at TFC was reported in 2003[4]. That work indicated that Dawn strain was not only more benign than the current *A. centrale* vaccine strain in terms of vaccine reactions, but also provided better protection against virulent field strains of *A. marginale*. In two trials to assess the virulence of this isolate, no animals showed clinical reactions, while 14% of the controls vaccinated with *A. centrale* required treatment according to preset trial criteria based on clinical symptoms (anaemia) and parasitaemia. When challenged with Australian isolates in four trials, steers previously exposed to Dawn *A. marginale* were almost completely protected against reinfection; none of the 27 animals in the four trials required treatment. In contrast, *A. centrale* vaccinated animals developed low parasitaemias and 3 of the 32 animals in the four trials were treated, as were 17 of the 25 unvaccinated controls.

In field trials conducted in 2010, involving nearly 800 animals (TFC unpublished), Dawn strain proved highly infective at both a similar dose to the current *A. centrale* dose and one-tenth that dose, even

with longer pre-manufacture blood storage (up to three days) and longer vaccine storage (up to six days). These results suggested the possibility of extending the shelf-life of the trivalent tick fever vaccine if Dawn strain *A. marginale* could be successfully incorporated into the vaccine as the *Anaplasma spp* vaccine strain.

Attempts have also been made to assess the ability of *R. (B.) microplus* to transmit Dawn *A. marginale* in pen trials at TFC[4]. Both attempts were unsuccessful; and whilst this does not allow the conclusion that it is not transmissible by *R. (B.) microplus*, these results certainly indicate that it is not easily transmitted.

Dawn *A. marginale* is currently available under Australian Pesticides and Veterinary Medicines Authority (APVMA) Minor Use Permit 11755 as a monovalent frozen vaccine. It has been used successfully and without adverse reactions since 2004 on two properties where *A. centrale* vaccination had failed to prevent anaplasmosis. On one property it is used as a monovalent "booster" vaccination after initial standard trivalent tick fever vaccine, whilst on the other it is used as part of the primary vaccination program in combination with *Babesia bovis* and *Babesia bigemina*.

2. Objectives

Given the mild nature of Dawn strain *A. marginale*, the advantages it offers in terms of better protection of cattle against anaplasmosis and the potential to extend the shelf life, the intention of this project was to further evaluate this strain in line with APVMA requirements for registration of immunobiologicals. The aim was to replace *A. centrale* with Dawn strain *A. marginale* in the registered chilled and frozen tick fever vaccines.

Requirements for registration with APVMA, based on a Minor Use Permit being already in place for this organism, focused on a major change to seed strain for our current vaccine, with the following three components:

- Application overview
- Module 2.2 – Chemistry and Manufacture
- Module 8.3 – Safety and Efficacy

From this the following project objectives were developed in relation to Module 8.3:

1. To further evaluate the safety and efficacy of Dawn strain *A. marginale* in line with the requirements to achieve full registration of this strain with APVMA as a component of trivalent chilled and frozen tick fever vaccines
2. To validate the shelf life of Dawn strain *A. marginale* in chilled and frozen tick fever vaccines
3. To further investigate the tick transmissibility of Dawn strain *A. marginale*
4. To develop a marker which allows discrimination of vaccine and field strains of *A. marginale*

3. Methodology

MLA is committed to investing in top quality scientific research, performed by suitably qualified, experienced and registered researchers and organisations. In experiments that involve livestock, MLA acknowledges that such research needs to be done under the auspices of a recognised Animal Care and Ethics Committee (AEC). The responsibility for obtaining AEC approval lies with the researcher. MLA has in the past not specifically asked for evidence that such AEC approval had indeed been obtained.

3.1 Confirmation of dose

A small trial involving 21 steers (three groups of seven animals each) was conducted to clarify the dose of Dawn *A. marginale* to be used in the main vaccine trials. To mimic the current vaccine production procedure using *A. centrale*, three small batches of trivalent tick fever vaccine were made using Dawn *A. marginale* blood which had been stored for one day after collection. The three batches contained Dawn strain *A. marginale* at three doses rates (4×10^7; 1×10^7 or 0.1×10^7 per dose respectively). Each of the batches also contained *B. bovis* (1×10^7 per dose) and *B. bigemina* (2.5×10^6 per dose). The vaccine was stored in the refrigerator for four days after manufacture, to mimic the current limit of vaccine shelf-life prior to administration. Samples were collected from the steers at appropriate time periods post-vaccination to detect infection by microscopic examination of blood smears, real-time polymerase chain reaction (qPCR), and determination of antibody levels. Details are found in Appendix MS2.1 (MS2 = Milestone 2 Report)

3.2 Safety and efficacy of Dawn strain *A. marginale* vaccine

3.2.1 Safety, infectivity and virulence

Target animal safety and efficacy studies were conducted in accordance with APVMA guidelines for registration of a new strain of organism as an immunobiological product. Evaluation of efficacy included both infectivity (that is, a patent *Anaplasma spp* infection was established and an immune response developed following vaccination); and ability to withstand challenge from virulent Gypsy Plains strain *A. marginale*.

Standard trivalent chilled and frozen Combavac 3in1 tick fever vaccines containing *A. centrale*, and previously manufactured monovalent Dawn strain *A. marginale* frozen batches M001 and M004, were used as controls. One other control group received bivalent *B. bovis* and *B. bigemina* vaccine only. The experimental vaccines were produced by a manufacturing process similar to that of the registered products. The chilled trivalent vaccines contained 1×10^7 Dawn strain *A. marginale* per dose as a substitute for *A. centrale*; and the experimental frozen Combavac 3in1 also contained Dawn strain *A. marginale* instead of *A. centrale*.

Three batches each of trivalent chilled and frozen vaccine containing Dawn strain *A. marginale* were tested. Safety (including 10x overdose – Group 3) and infectivity were evaluated in vaccine batches that are potentially most virulent (that is, vaccine made and used on the day of blood collection). Efficacy (infectivity) was evaluated in vaccine batches at the end of the shelf-life (vaccine made one day after blood collection and used four days after that). Vaccines containing Dawn strain *A. marginale* were compared with vaccines containing *A. centrale* as the control product. An experimental vaccine to test "reversion to virulence" after syringe passage of Dawn strain *A. marginale* through two non-splenectomised steers was also included (Group 12).

To obtain Dawn *A. marginale* parasites for chilled vaccine production, a schedule for stabilate inoculation and weekly passage of *A. marginale*-infected blood was established to mimic the current *A. centrale* schedule for production of standard trivalent vaccine. *A. marginale*-infected blood was collected to make 3 batches of chilled and frozen vaccine from Passage 1, Passage 4 and Passage 6 calves; this represented the beginning, middle and end of the passage process. Chilled experimental Dawn *A. marginale* vaccine at various passages was administered on the day of manufacture or four days after manufacture, and after nil or one day blood storage pre-manufacture (representing fresh and stored vaccine) to assess differences in safety, infectivity and virulence.

To make some assessment of reversion to virulence (albeit by syringe passage, not tick transmission), blood harvested from a splenectomised calf which had been inoculated with Dawn *A. marginale* stabilate V34 was passaged through two nonsplenectomised steers and then back into a splenectomised calf, from which vaccine was subsequently made (see Appendix MS2.2 - Group 12).

The groups were vaccinated on different dates determined by passage number (passages 1, 4 and 6) and date of manufacture (determined by blood and vaccine storage). Vaccination occurred in three subgroups: Groups 1–8, 9–11 and 12–17. Groups 1–8 were used to assess safety, infectivity and virulence; Groups 9–11 infectivity only and Groups 12–17 infectivity and virulence. Details of the vaccine groups are listed in Table 1, Appendix MS2.2.

A schedule of collection of samples for blood smear examination, qPCR and serology; packed cell volume (PCV) to measure degree of anaemia; measurement of temperature; and examination of the injection site is described in Appendix MS2.2.

A number of criteria were used in assessment of the safety, infectivity and virulence of the vaccines. These included fever after vaccination and during the *Babesia* spp reaction periods; evidence of any local reaction at the site of vaccination; evidence of vaccine organisms in blood smears and estimation of the percent infected erythrocytes (PIE); detection of vaccine organism deoxyribonucleic acids (DNA) by qPCR and of antibody by serology; and PCV depression to indicate degree of anaemia caused by the *Anaplasma spp* infection.

3.2.2 Challenge study

Sixty-five Hereford steers (approximately 20 months of age) previously used in the safety, infectivity and virulence trial (see 3.2.1 above; and Appendix MS2.2) were selected based on vaccination history and evidence of infection after vaccination. Seven steers were allocated to each of eight groups, based on whether they had been vaccinated with *A. marginale* or *A. centrale*, frozen or chilled vaccine, or the Dawn *A. marginale* passage number (1, 4 or 6) that the original vaccine had been derived from; with an extra group of nine animals included where infectivity of the *A. marginale* vaccine during the original vaccine infectivity trial was not certain. One group had never received *Anaplasma spp* vaccine and thus remained fully susceptible to challenge.

While it had been the original intention to challenge *A. marginale* groups which had been vaccinated using Passage 1 and Passage 6 derived *A. marginale* organisms, less than optimum infectivity of some of the vaccines necessitated recombination of the groups for the challenge trial.

These 65 steers were inoculated intravenously with a challenge dose of 1 x 10^8 virulent Gypsy Plains strain *A. marginale* approximately three and one half to five months after vaccination. Monitoring of the response to challenge was principally by microscopic examination of blood smears for *A. marginale* organisms; and measurement of PVC depression to show the degree of anaemia resulting from the challenge. Control animals were also monitored by qPCR and serology. Treatment criteria for clinical illness associated with the virulent *A. marginale* infection was a PCV less than or equal to 15%; a PCV less than 20% with a parasitaemia greater than 15 per high power field; or severe clinical distress regardless of the above parameters. For further detail, see Appendix MS2.3.

3.3 Tick transmissibility of Dawn strain *A. marginale*

The method of infection and number of cattle was based on that used previously by TFC staff in tick transmission studies with Gypsy Plains strain *A. marginale*[6]. Forty steers were purchased from a

tick-free area of Queensland for this trial which was conducted at Mutdapilly Research Station after an initial induction period at TFC prior to tick infestation. Ten of these steers were infected with both Dawn strain *A. marginale* and tick larvae from an acaracide susceptible strain of *R. (B.) microplus*. These infected "donor" steers were then run in a paddock together with a further 30 naïve steers; the donor and naïve steers were mixed on the ninth day after *A. marginale* infection, and from 0 to 4 days after tick infestation. The 30 naïve steers were monitored in the ensuing months for evidence of transmission of Dawn strain *A. marginale* up to 104 days after first exposure to the tick-infested donor steers. Samples were collected at appropriate time periods for microscopic examination of blood smears for *A. marginale*, qPCR, and determination of antibody levels. Further details are found in Appendix MS2.4

3.4 Marker to identify Dawn strain *A. marginale*

Two candidate genes, Akyrin repeat B gene (AnkB) and AM415 gene, were selected as potential diagnostic markers for strain differentiation by Dr Kelly Brayton of Washington State University, USA (unpublished)[7]. When compared with US and Puerto Rican strains, the Australian Gypsy Plains *A. marginale* AnkB gene only had a 96-97% similar sequence identity. Further Dr Brayton revealed the missing AM415 gene region in Dawn *A. marginale* compared to Australian Gypsy Plains strain *A. marginale* and thus selected it as a candidate. The DNA regions of both candidate genes were amplified by PCR, then sequenced and analysed for differences. The AnkB gene was assessed for differences in single nucleotide polymorphisms (SNP). By contrast, the presence or absence of AM415 gene was analysed; and Dawn strain *A. marginale* results compared to *A. centrale* and 15 additional Australian *A. marginale* isolates. For further detail, see Appendix FR1 in this report (Section 6).

4. Results

4.1 Confirmation of dose

This trial confirmed that a dose of 1 x 10^7 organisms was sufficient to result in patent infection and antibody production after vaccination; and corroborated results from previous trial work (see Appendix MS2.1).

Benign *Theileria spp* were found on examination of blood smears from all animals

4.2 Safety and efficacy of trivalent tick fever vaccine containing Dawn strain *A. marginale* vaccine

4.2.1 Safety

The safety of vaccines which include Dawn strain *A. marginale* was evaluated in the period up to 4 days after vaccination; and subsequently through the *Babesia spp* reaction period of the trivalent vaccine from Days 7 to 21 (TFC data). This was assessed through maximum temperature, temperature rise over 3 days from Day 0 to 21 and maximum PCV depression from Day 7 to 21. There were no observable adverse systemic reactions in any animals and none required treatment.

Analysis of the *Babesia spp* reaction period is not further reported here as we were primarily interested in the response to the *Anaplasma* spp. component of the vaccine. For further detail, see Appendix MS2.2.

Benign *Theileria spp* were found on examination of blood smears from all animals.

4.2.2 Infectivity and virulence (*Anaplasma spp* reaction period)

The known vaccine reaction period for *A. centrale* is 30 to 60 days post vaccination (TFC data) and intensive monitoring was conducted in that period. Dawn strain *A. marginale* infectivity was poor and variable. Based on microscopic examination, qPCR and serology results, infectivity of Passage 1 chilled and frozen trivalent vaccine containing Dawn strain *A. marginale* (Groups 1–3 and 8) was 57–71%, compared to 86% for monovalent *A. marginale* frozen vaccine and 100% for vaccines containing *A. centrale.* Even Group 3, which had received ten times the dose to assess safety of overdose, had poor infectivity (57%). Infectivity for Passage 6 vaccine (Groups 13, 14 and 17) was only 14–57%, compared to 71% for both monovalent *A. marginale* frozen vaccine and standard chilled *A. centrale* vaccine. No control groups were used for Passage 4 (Groups 9–11); infectivity of the Dawn strain *A. marginale* was 100% for trivalent frozen vaccine and 71% for the trivalent chilled vaccine at standard and 1/10th of standard dose.

The criteria to assess virulence for Dawn *A. marginale* was PCV depression from Day 30 to 60 post-inoculation, expressed as the mean maximum percentage depression below pre-inoculation values. Monitoring started on Day 28, based on the results from the dose confirmation trial (see Appendix MS2.1).

Virulence was assessed using only steers which were considered to have been infected with Dawn strain following vaccination. Steers which were not considered infected were not included; except for the bivalent *Babesia spp* vaccine controls (Group 4).

The mean maximum PCV depression for the standard dose vaccine groups (Passage 1, Groups 1–2 and 5–8) containing either *A. marginale* or *A. centrale* ranged from about 16% to 27%. There was a significant difference of maximum PCV depression between Group 2 (standard fresh chilled *A. marginale*) at 27% and Group 3 (10x overdose) at 12%. No steers required treatment during the *Anaplasma spp* reaction period. Poor infectivity in Groups 12–17 (reversion to virulence and Passage 6 vaccines) also precluded any useful analysis of virulence in these groups.

4.2.3 Efficacy in challenge studies

Evidence of parasitaemia on blood smears (especially in the unvaccinated controls and *A. centrale* groups) was much less then expected from previous trial work. This apparent lack of infectivity and virulence of Gypsy Plains strain was not anticipated and made interpretation of results problematic.

There was no significant difference between any of the groups for mean maximum PCV depression or mean maximum *A. marginale* PIE (percent infected erythrocytes), although the unvaccinated controls had one animal which met treatment criteria (see Appendix MS2.4). The *A. marginale* chilled and frozen trivalent and frozen monovalent vaccine immunity (assessed through PCV depression, PIE and numbers requiring treatment) was similar to that of *A. centrale* trivalent and Combavac 3in1 vaccines.

Despite smear results, all the unvaccinated control steers (Group 4) which had not previously received *Anaplasma spp* vaccine seroconverted*,* and this was corroborated by positive PCR results (see Appendix MS2.4). Sub-inoculation of blood from each of four of the Group 4 control steers into

four splenectomised calves also demonstrated that these steers were infected with Gypsy Plains strain *A. marginale.*

4.3 Tick transmissibility of Dawn strain *A. marginale*

Both cattle tick infestation and Dawn strain *A. marginale* infection established in the ten donor steers, based on smear examination, qPCR and serology results. Maximum parasitaemia detected in eight of these steers up to Day 32 was estimated from about 0.4% to 4% PIE.

No evidence of *A. marginale* infection was detected in blood smears collected from the 30 naïve steers twice weekly between Day 38 and Day 76 after first mixing with the donor steers. At Day 76 after mixing with the donor steers, based on serology and qPCR results, there was also no evidence of *A. marginale* infection; this was confirmed again at Day 104.

Benign *Theileria spp* were found on examination of blood smears from all animals.

4.4 Marker to identify Dawn strain *A. marginale*

The AnkB gene showed identical sequences for both Gypsy Plains and Dawn Australian *A. marginale* strains, with no single nucleotide polymorphisms (SNPs) identified. The AnkB gene was thus not pursued as a diagnostic marker.

The absence of the AM415 gene in Dawn strain *A. marginale* was confirmed by the lack of amplification by PCR assay comparable to the negative control. The successful amplification of the AM415 gene resulted in a 1,027bp product that was present in *A. centrale* and the 15 field isolates. It is suggested that the AM415 gene is a gene duplication of AM414 due to a high level of sequence identity between the two, and its absence does not affect virulence function (Brayton et al. In A Akim Kerudin et al., unpublished data).

Therefore the AM415 PCR assay can discriminate Dawn *A. marginale* from *A. centrale* and other *A. marginale* field isolates.

5. Discussion and Conclusions

5.1 Safety

Safety was measured as any adverse event (fever, site reactions, or clinical illness) in the period immediately following vaccination; and response to ten-times overdose. The vaccine does not contain any adjuvants and has a neutral pH; therefore it is not surprising that there were no adverse systemic reactions associated with inclusion of *A. marginale* and no differences observed between *A. marginale* and *A. centrale* vaccine groups; or indeed even the ten-times overdose group. Any reaction to the vaccine therefore is likely to be directly associated with the virulence of the vaccine organisms themselves, and this only becomes evident in reaction periods 7 to 21 and 30 to 60 days post-vaccination for *Babesia spp* and *Anaplasma spp* respectively.

5.2 Infectivity and virulence

In this trial, infectivity of the Dawn stain *A. marginale* trivalent chilled and frozen vaccines was poor. Across all three batches (made from Passage 1, 4 and 6), *A. marginale* infectivity ranged from 14–71%, except for Passage 4 frozen trivalent vaccine which was 100% infective. This is difficult to

explain. In the dose confirmation trial conducted as part of this project in 2011, the infectivity ranged from 85–100% with vaccines which had been made from blood stored for 1 day and then the vaccine stored for a further 4 days prior to use. In field trials conducted in 2010, prior to this project, even with pre-manufacture blood storage for up to 3 days and postmanufacture vaccine storage of up to 6 days, the Dawn strain *A. marginale* vaccine induced antibodies in most animals at one-tenth the standard dose. However, whilst not shown in the data here, the Dawn strain *A. marginale* stabilates used to inoculate splenectomised calves for vaccine production have not always initiated sufficient parasitaemia in these calves to produce vaccine (R. Bock, pers comm; TFC data, this trial). The possibility that infectivity in the vaccine declines with continuing passage in splenectomised calves should also be considered; although this was certainly not evident in the splenectomised calves used as *A. marginale* donors for vaccine production.

Given that fever is not a consistent feature of *A. marginale* infection, virulence was measured by PCV depression and an estimate made of the parasitaemia during the reaction period 30 to 60 days post-vaccination. Overall there was no statistically significant difference in virulence between vaccines containing *A. marginale* (chilled and frozen) or *A. centrale* (trivalent and Combavac 3in1) vaccines in Groups 1–3 and 5–8; although the PCV depression in the vaccine groups was greater than the unvaccinated controls (Group 4 – no *Anaplasma spp*). The PCV depression in the ten-times overdose group (Group 3) supported previous observations that the anaemia after infection is not a function of dose[8]. The poor infectivity of *Anaplasma spp* but particularly the *A. marginale* vaccines in Groups 13–17 made interpretation of results of the vaccines prepared from the Passage 6 calf difficult.

5.3 Efficacy

The failure to detect any parasitaemia in blood smears in six of the seven unvaccinated steers after inoculation with Gypsy Plains strain *A. marginale* was not expected. Only one steer from this group required treatment to control the *A. marginale* infection. It was subsequently shown by a combination of smear examination, qPCR, serology and sub-inoculation of blood into splenectomised calves that all seven unvaccinated controls were in fact infected. It is therefore surprising that it did not produce clinical effect consistent with its previous history of virulence (TFC unpublished)[4]; and this is not easily explained.

In this challenge subclinical mean maximum PCV depression, negligible mean maximum PIEs and animals requiring treatment might suggest either immunity imparted by vaccines, poor virulence or infectivity of Gypsy Plains strain *A. marginale*, an unforeseen consequence of *Theileria spp*. infection, or an unforseen animal effect. These factors conceal (in this trial at least) any differences that may exist in the level of protection imparted by Dawn *A. marginale* vaccine compared to *A. centrale*.

5.4 Tick transmissibility

Using similar methodology to a previous study using Gypsy Plains *A. marginale*[6], there was no evidence in this trial that Dawn strain *A. marginale* was transmitted by *R. (B.) microplus*. This is consistent with the findings previously described in pen trials during the initial assessment of Dawn strain[4]. It appears that Dawn *A. marginale* is, at the very least, not readily transmitted by *R. (B.) microplus* although more evidence would be required to exclude transmission completely.

5.5 Marker to differentiate Dawn strain *A. marginale*

Dr Ala Lew-Tabor and staff from University of Queensland, Queensland Alliance for Agriculture and Food Innovation (UQ, QAAFI, unpublished data 2012) developed a PCR assay that can successfully distinguish Dawn strain *A. marginale* from *A. centrale* and other *A. marginale* field isolates.

The finding that Dawn strain *A. marginale* is poorly tick transmissible is important, as further development of this molecular method offers a means to identify vaccinated cattle and investigate reported failures of any future Dawn *A. marginale* inclusive tick fever vaccine. The MSP5 cELISA is the recommended method for confirming *Anaplasma* infection because of the recurring low level parasitaemia that is undetectable by PCR[9]. Ultimately, development of a Dawn *A. marginale*-specific cELISA would make way for a more complete diagnostic interpretation.

5.6 *Theileria spp* infection

In recent years, outbreaks of theileriosis have caused major concern in NSW and Victoria[10,11]. To alleviate confusion about nomenclature and to differentiate the aetiological agent from exotic theileriosis caused by *Theileria parva* and *T. annulata*, the disease here is designated by the Theileriosis Working Group as "bovine anaemia caused by *Theileria orientalis*" (G, Bailey, NSW DPI, pers comm.). Relatively non-pathogenic *Theileria spp* (noted then as *T. buffeli*) are very prevalent in Queensland cattle herds[12] but infection is rarely associated with clinical disease (TFC unpublished). An effective registered treatment for *Theileria spp*. is not currently available in Australia.

Theileria spp are endemic on the TFC property where these vaccine trials were conducted. The trial commencement date was delayed for three months to allow the *Theileria spp* infection to become more chronic and thus presumably interfere less with the PCV measurements. It is difficult to ascertain just how that infection interacted with the trial results, given that PCV depression is a function of the initial *Theileria spp* infection. Although problematic, we believed it was useful to assess the vaccines under mimicked field conditions. Also there was no obvious effect of *Theileria spp* infection on infectivity in the original dose confirmation trial in 2011 (see Appendix MS2.1).

However, previous data obtained from 12-18 month old Hereford steers, comparing *Theileria spp*-infected to *Theileria spp*-free cattle, showed that maximum *A. marginale* parasitaemia (33% of *Theileria spp*-infected cattle had maximum parasitaemia of <0.1% compared to minimum parasitaemia observed in *Theileria spp*-free cattle of 7%), clinical anaemia and number of steers requiring treatment (50% versus 90% respectively) were significantly affected by *Theileria spp* infection status [13]. As no serological cross reactivity exists between *A. marginale* and *Theileria spp,* the effect is assumed to involve non-specific cell mediated immunity.

There are two further aspects of this to note. Firstly, despite the findings mentioned above[13], concern has not been expressed in the past about the effect of *Theileria spp* infection on *A. centrale* or *A. marginale* infection in trials conducted over the years at TFC. However, in trials conducted as part of the breed susceptibility project in 2010 (MLA B AHE.0050), 65 of 79 previously naïve animals developed a parasitaemia post-inoculation with Gypsy Plains strain *A. marginale*, and all 65 required treatment by Day 22 post inoculation; but interestingly no parasitaemia was detected in 14 animals post-inoculation (TFC unpublished). Secondly, the property where we carried out the Dawn *A. marginale* vaccine trials in 2010 with impressive results was *Theileria spp* PCR negative when tested as part of a small survey in Queensland (G.

Eamens, NSW DPI, pers comm.); and so there would have been no effect of *Theileria spp* on that property.

5.7 Diagnosis of *A. marginale* infection

Diagnosis of *A. marginale* infection was by analysis of a combination of light microscopy of blood smears, qPCR, VMRD *Anaplasma spp* cELISA and limited subinoculation of naïve splenectomised calves to confirm some of the inconclusive results. The VMRD *Anaplasma spp* cELISA has replaced the Card Agglutination Test (CAT) (described as a "capricious" test by TFC staff) for diagnosis of *A. marginale*. Now that we have gathered some data from this project on the VMRD *Anaplasma spp* cELISA, further analysis of the sensitivity and specificity is required to validate its use under Australian conditions.

5.8 Conclusions and recommendations

Dawn strain *A. marginale* was assessed as a potential vaccine candidate strain for inclusion in trivalent tick fever vaccines as a replacement for *A. centrale*. The vaccines were assessed in lines with the guidelines outlined in APVMA's requirements for registration of immunobiologicals (Module 8.3: Safety and Efficacy).

There is some corroboration of a previous study where Dawn strain *A. marginale* proved to be as safe as *A. centrale* and no more virulent. Molecular studies have identified a missing gene in Dawn strain *A. marginale* which may be useful to distinguish it from other field isolates; and the poor tick transmissibility of Dawn strain *A. marginale* has been confirmed.

However, poor infectivity of Dawn strain *A. marginale* in the experimental trivalent vaccines is not easily explained given previous results; and apparent lack of virulence in the challenge trial using Gypsy Plains strain *A. marginale* has made any interpretation of efficacy of the vaccines difficult. Reasons for poor infectivity and the effect of *Theileria spp* infection on response to *A. marginale* infection need to be further investigated; and the challenge study needs to be repeated before consideration is given to registration of Dawn *A. marginale* as a vaccine strain.

Recommendations:

1. Reasons for poor infectivity of Dawn strain *A. marginale* and apparent lack of virulence of Gypsy Plains strain *A. marginale* in this trial need to be further investigated.
2. The effect of *Theileria spp* infection on subsequent *A. marginale* infection needs further evaluation.
3. Cut-off points and sensitivity and specificity of the VMRD *Anaplasma spp* cELISA need to be discussed with the manufacturer of the test kit; and further evaluation conducted under Australian field conditions.

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7. Appendix

7.1 Appendix FR1: Dawn *A. marginale* PCR assay development

The tick fever vaccine imparts protection against anaplasmosis through *Anaplasma centrale.* The only known tick vector of *A. centrale*, *Rhipicephalus simus*, is not present in Australia which precludes any biological transmission (Potgieter & van Rensburg, 1987). Therefore the presence of *A. centrale* or *A. centrale*-specific antibodies is evidence of vaccination (Molloy et al., 2001).

Molloy et al. (2001) developed an *A. centrale*-specific competitive inhibition enzymelinked immunosorbent assay (cELISA) to serologically indentify vaccinated cattle. Their study revealed 100% sensitivity and specificity of the ELISA in experimentally infected cattle with *A. centrale* and *A. marginale* respectively. In addition, the cELISA consistently detected cattle in the carrier stage of

infection; and antibodies up to 9 years after vaccination with no evidence of reduced sensitivity (Molloy et al., 2001).

The ELISA is invaluable for investigations of tick fever vaccine efficacy breakdowns and untoward reactions (Molloy et al., 2001). It is used to confirm vaccination status of cattle suffering from babesiosis, as well as to quantify immunity developed against *A. centrale* in cases of anaplasmosis. Additional advantages of this serological assay include ability to test large numbers of affected cattle, rapid turnaround of results and relative inexpense.

Incorporation of Dawn strain *A. marginale* into the trivalent vaccine would result in the inability to identify vaccinated animals or to differentiate between vaccine and field isolates of *A. marginale* thus placing reliance on other diagnostic tests. Lew et al. (2002) developed the *msp*1α polymerase chain reaction (PCR) assay that specifically amplified *A. marginale*. This enabled comparison of *A. marginale* isolates during recurring parasitaemia. However, evidence that the *msp*1α genes in Australian *A. marginale* isolates are a largely conserved group makes discrimination of *A. marginale* isolates from different outbreaks in Australia difficult (Lew et al., 2002).

In 2012, Ammielle Akin Kerudin, Dr Ala Lew Tabor and Dr Jess Morgan from the University of Queensland, Queensland Alliance for Agriculture and Food Innovation (UQ, QAAFI, unpublished data) developed a PCR assay that can successfully distinguish Dawn strain *A. marginale* from *A. centrale* and other *A. marginale* field isolates. The remainder summarises their research methodology, results and discussion.

Two candidate genes, namely Akyrin repeat B gene (AnkB) and AM415 gene, were selected as potential diagnostic markers for strain differentiation by Dr Kelly Brayton of Washington State University, USA. The Australian AnkB gene derived from Gypsy Plains strain *A. marginale* had only had a 96-97% similar sequence identity when compared with strains from USA and Puerto Rico. The AnkB gene was selected based on this difference in sequence and because of potential for variation in single nucleotide polymorphisms (SNP) between Australian isolates. Further Dr Brayton revealed the missing AM415 gene region in Dawn *A. marginale* compared to Australian Gypsy Plains strain *A. marginale* isolate and thus selected it as a candidate.

The DNA regions of both candidate genes were amplified by PCR, then sequenced and analysed for differences. The PCR oligonucleotide primers were provided by Integrated DNA Technologies and the AM415 primers were designed by Dr Brayton's research group.

The AnkB gene was sequenced, aligned with Gypsy Plains strain *A. marginale* (previously sequenced by Dr Brayton's research group) and assessed for differences in SNPs. The 134bp AnkB gene showed identical sequences for both strains with no SNPs identified, and the AnkB gene was thus not pursued as a diagnostic marker.

The presence or absence of AM415 gene was analysed; and Dawn strain *A. marginale* results compared to *A. centrale* and 15 additional Australian *A. marginale* isolates. The absence of the AM415 gene in Dawn strain *A. marginale* was confirmed by the lack of amplification by the PCR assay comparable to the negative control. The successful amplification of the AM415 gene resulted in a 1,027bp product that was present in *A. centrale* and the 15 field isolates. It was proposed that the absent AM415 gene does not affect the virulence function of Dawn strain *A. marginale* as it is compensated by the preceding gene in the genome, AM414. It is suggested that the AM415 gene is a gene duplication of AM414 due to a high level of sequence identity between the two (Brayton et al., in A. Akim Kerudin et al., unpublished data).

Therefore the AM415 PCR assay can discriminate Dawn *A. marginale* from *A. centrale* and other *A. marginale* field isolates.

Several trials have now indicated that Dawn strain *A. marginale* is poorly transmitted by ticks, if it is at all (Bock et al, 2003; Appendix MS2.4, this project). This is an important feature if this strain was ever to be incorporated into trivalent tick fever vaccine as a replacement for *A. centrale.* With further development, this molecular method offers a means to identify vaccinated cattle and investigate reported failures of a Dawn *A. marginale* inclusive tick fever vaccine. Further investigation of the sequence of the missing gene region in Dawn *A. marginale* was recommended to increase reliability of the diagnostic assay. Future research would be focused on adapting the assay to a more sensitive and user-friendly real-time PCR format. According to Lew et al. (2002) MSP5 ELISA is the recommended method for confirming *Anaplasma* infection because of the recurring low level parasitaemia that is undetectable by PCR. Ultimately then, development of a Dawn *A. marginale*specific ELISA would make way for a more complete diagnostic interpretation.

7.1.1 Appendix FR1: References

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