final report

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Development of a vaccine against Annual Ryegrass Toxicity

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Development of a vaccine against Annual Ryegrass Toxicity

Australian Wool innovation Project WP001

June 2009

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Executive summary

The Plant Toxins Research Group, CSIRO Livestock Industries, have previously developed a prototype vaccine based on modified tunicamycins and Australian foetal calf serum carrier proteins that successfully protected sheep from the clinical effects of Annual Ryegrass Toxicity in laboratory trials. A commercial assessment of the current vaccine concluded that a commercial partner could become involved as soon as formulation and administration aspects were resolved. This project was designed based on these recommendations, with commercially important parameters such as vaccine dose, regime, antibody kinetics, vaccine stability and safety investigated in an 18 month project using 21 groups of merino sheep.

The vaccine has been robust in performance. Sheep tolerated the vaccine well, with a commercially acceptable level of injection site reactions. There seems to be high degree of tolerance to differing doses, timing and storage of the vaccine. There was little significant difference in antibody kinetics among the groups and titres produced were consistently above what is thought to be protective levels based on previous successful challenge studies. All results in this study were positive in terms of the future progression of this vaccine through to a commercial product.

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1 INTRODUCTION

1.1 Background

ARGT impacts upon animal health, welfare and productivity in addition to farm economics and producer morale. In the ARGT-endemic area of Western Australia alone, 1997 Australian Bureau of Statistics figures indicate a presence of about 16 million sheep and 300,000 cattle. In normal ARGT seasons the average number of sheep deaths in WA is around 30,000. In severe ARGT seasons there have been as many as 88,168 reported deaths from ARGT. Non-lethal effects of ARGT include reduced wool production and quality; abortion; poor, post-intoxication reproduction; longer finishing time for lambs of exposed ewes.

A successful vaccine will eliminate or reduce the effects of ARGT such as management problems, emotional costs to wool growers and livestock producers, clinical toxicity and reduction of wool growth, wool fibre diameter and total wool volume. It will reduce the potential for animal welfare concerns related to the dramatic death and other clinical effects to adversely impact domestic and international markets. A successful vaccine may also address human food safety concerns by preventing accumulation of the ARGT toxins (corynetoxins) in animal tissues by increasing the rate of clearance of the toxins from the tissues.

1.2 Rationale

Using CSIRO-patented technology (Australian Patent application No 66010/98), the Plant Toxins Research Group of the CSIRO Livestock Industries developed a vaccine, based on modified tunicamycins and Australian foetal calf serum (FCS) carrier proteins, that successfully protected sheep from the clinical effects of Annual Ryegrass Toxicity (ARGT) in laboratory trials. A commercial assessment of the current vaccine, commissioned by Meat and Livestock Australia (MLA) and conducted by Baron Strategic Services Pty Ltd., concluded that:

“the ARGT vaccine could be commercially viable notwithstanding the geographically limited market. Some additional development work is recommended prior to commercialisation. It is also recommended that the possibility of using a recombinant replacement for tunicamycin be explored.” In addition: “commercialisation requires finalisation and optimisation of the formulation and dose regime, confirmation of raw material sources, optimising production, establishing shelf life, efficacy and safety, and preparing a regulatory dossier. A commercial partner could become involved as soon as formulation and administration aspects were resolved.”

Thus, this project will define commercially important parameters for the experimental ARGT vaccine. Criteria such as the optimum (or near optimum) amount of immunogenic conjugate to be included in the vaccine, the vaccination protocol (how many booster injections are required after the first vaccination), the rate of production and decline of corynetoxin antibodies, the character of induced antibodies (IgM, IgG), stability of the vaccine during storage and formulation variables such as dose volume and injection site reactions will be defined.

1.3 Objectives

The Project is expected to result in a vaccine product which will be attractive to a commercial partner for further development into pen-based challenge studies, field trials and the accumulation of registration data.
What we want to know:

- The optimum time for giving the booster injection of the ARGT vaccine after the first injection. Timing the second injection for just after the peak of antibody titre following the primary injection is expected to result in the best boost to corynetoxin antibody levels.
- The optimum concentration of immunogenic conjugate required to stimulate the highest and most stable (persistent) level of corynetoxin antibodies i.e., the kinetics of the antibody production
- Whether a secondary booster injection is required

- Which of two volumes of vaccine formulation produces the better antibody titre kinetics and the best memory effect after 12 months
- Which of 3 immunogenic conjugate levels provides the most stable vaccine when stored for 6 months and 12 months.
- Do antibody isotypes induced conform to that expected of a successful vaccine.

This investigation, planned to be completed in 24 months, will be divided into three Studies:

- The first study will compare immunogen levels, quantitate the kinetics of corynetoxin antibody production and decline following different schedules of primary (V1), first booster (V2) and second booster (V3) injections, determine the character of induced antibodies and establish the best memory response following re-vaccination after 12 months.
- The second study will determine, using a selected immunogen level and vaccination schedule determined in the first study, the effect of the immunogen formulated into a vaccine dose volume of 1 ml. The second study will also determine the kinetics and character of the antibody response, especially after the 12 month re-vaccination, using a V1 – V2 protocol only, i.e., leaving out the V3 from the most successful protocol determined in the first study.
- The third study will establish the stability of the vaccines stored for 6 months and for 12 months.

The vaccination protocol that has the best “industry-friendly” antibody titre characteristics can then be used in future challenge studies to prove efficacy under pen and field conditions.

2 STUDY DESIGN AND RESEARCH STAFF

2.1 Study Personnel and Locations

2.1.1 Study Personnel

Table 1 Study Personnel

Name	Responsibilities
Steven Colegate	Initial Project Leader and interaction with funding body and Industry groups. Dr Colegate ceased with CSIRO on 30 June 2008.
Neil Anderton	Neil is the team's chemist and was responsible for the preparation of the hapten and conjugate, and determination of the vaccine's physical and chemical stability characteristics.
Yu Cao Agnieszka Michalewicz	Both Yu and Agnieszka are expert in the application of the corynetoxin ELISA and RIA and were responsible for preparing and assessing the sera for corynetoxin antibodies and isotyping the antibodies. This was very labour intensive due to the number of sera collected.
Peter McWaters	Peter advised on the immunological aspects of the project
Phil Stewart	Phil was the team's biochemical toxicologist. He supervised the initial vaccine preparation and vaccinations.
Mark Ford	Veterinarian: final arbiter on sheep health decisions and clinical assessments. Appointed as Project Leader in June 2008.
Sandy Matheson	Werribee Animal Facility manager. The team is indebted to Sandy's skill at coordinating all aspects of the sheep work in the study.
Noel Collins, Chris Darcy	In addition to Sandy, Noel and Chris were responsible for the sheep work at Werribee, including the large number of blood collections required.

2.1.2 Study Locations

2.1.2.1 Animal Facility

CSIRO Livestock Industries
Werribee Animal Facility (WAF)
6 South Rd
Werribee
VIC, 3030

2.1.2.2 Laboratory

CSIRO Livestock Industries
Australian Animal Health Laboratory
5 Portarlington Rd
Geelong
VIC, 3220

2.2 Basic Study Design

2.2.1 Study Design Structure – 3 Studies

2.2.1.1 Study 1

- 3 vaccine doses, 5 vaccination regimes – 13 groups of 10 sheep each
- Vaccine doses: 0.25, 0.5, 1.0 mg/2mL dose
- Sheep received 3 vaccinations plus an annual booster, across 5 vaccination regimes.

Group 9, a “gold standard” group was included which mimics the dose and regime that was used in the earlier successful challenge trials with this vaccine.

The objectives Study 1 was designed to achieve are:

- The optimum time for giving the booster injection of the ARGT vaccine after the first injection. Timing the second injection for just after the peak of antibody titre following the primary injection is expected to result in the best boost to corynetoxin antibody levels.

- The optimum concentration of immunogenic conjugate required to stimulate the highest and most stable (persistent) level of corynetoxin antibodies i.e., the kinetics of the antibody production
- In association with Study 2, which of two volumes of vaccine formulation produces the better antibody titre kinetics and the best memory effect after 12 months
- Which antibody isotypes are produced?

2.2.1.2 Study 2

- Vaccine volume and booster requirements – 2 groups of 10 sheep each
- Vaccine dose of 0.25mg/1mL (compared with a 2mL volume previously used)
- 2 vs. 3 injection regime

The objectives this Study was designed to achieve are:

- Whether a secondary booster injection is required
- Which of two volumes of vaccine formulation produces the better antibody titre kinetics and the best memory effect after 12 months
- Which antibody isotypes are produced?

2.2.1.3 Study 3

- Stability of the vaccine stored for 6 and 12 months (6 groups of 10 sheep each)
- Vaccine doses of 0.25, 0.5, 1.0 mg/2mL dose
- Vaccines stored at 4^{o}C for 6 and 12 months prior to vaccinations commencing

The objective this study was designed to achieve is:

- Do the 3 immunogenic conjugate levels retain their activity when stored for 6 months and 12 months?

2.2.2 Animal Management

2.2.2.1 Source of sheep

Merino sheep (wethers) were sourced from a farm in Newstead, near Castlemaine, Victoria. They were acquired when approximately 9 months old and maintained on paddocks at the WAF until their participation in the study commenced at between 12 and 15 months of age.

Prior to commencement of the studies, sheep husbandry was in accord with normal industry practice (Table 2).

Table 2 Sheep Husbandry Timetable

Study	Date	Procedure
Study 1 130 sheep	April – May 2006	Born
		All vaccinated with 2 doses of Clostridial (5 in 1) vaccine on farm, with normal drenching and castration husbandry
	Jan 2007	Arrived at CSIRO WAF
	21.2.07	5 in 1 vaccine
	23.2.07	Intestinal parasite drench
	19.4.07	Jetted for fly protection
	15.8.07	5 in 1 vaccine
	10.3.08	Lice treatment
	14.6.08	Vitamin A,D,E injection (routine)
	6.8.08	Intestinal parasite drench

Study	Date	Procedure
Study 2, 3a 50 sheep	July – August 2006	Born
		All vaccinated with 2 doses of Clostridial (5 in 1) vaccine on farm, with normal drenching and castration husbandry
	May 2007	Arrived at CSIRO WAF
	22.10.2007	Intestinal parasite drench
	5.12.2007	Jetted for fly protection
	19.2.2008	5 in 1 vaccine
	14.5.2008	Vitamin A,D,E injection (routine)
	4.6.2008	Lice treatment
	6.8.2008	Intestinal parasite drench
Study 3b 30 sheep	April – May 2007	Born
		All vaccinated with 2 doses of Clostridial (5 in 1) vaccine on farm, with normal drenching and castration husbandry
	Jan 2008	Arrived at CSIRO WAF
	14.4.08	Intestinal parasite drench
	6.8.2008	Intestinal parasite drench
	9.9.2008	5 in 1 vaccine

2.2.2.2 Identification

Sheep were identified with an ear tag in each ear. The tag in the left ear was used as the main identification, with the right ear tag providing cross reference if required.

2.2.2.3 Housing and Feeding

Sheep were paddock housed on pasture at the CSIRO Werribee Animal Facility. Due to the low rainfall conditions over the duration of the study, supplementary feeding formed the majority of their feed. Supplementary rations consisted of sheep pellets, lucerne hay, oaten hay, pasture hay, grass silage and the grazing of young oat crops, depending on availability, price and sheep condition. Sheep remained in good body condition for the duration of the trial, which is a credit to the farm manager at Werribee.

2.2.2.4 Grouping

Sheep grouping was based upon their bodyweights. This was a semi-random approach with the aim of ensuring that the group mean bodyweights were very similar i.e., that no one group accidentally included a greater proportion of the heavier (or lighter) animals, for example.

2.2.2.5 Animal Ethics considerations:

All facets of the animal research were approved by the CSIRO AAHL Animal Ethics Committee. This included acceptance of the Experimental Protocol, all annual reviews and modifications.

The approved protocol is included in Section 5.1.

2.2.2.6 Animal Health

In general the sheep remained in excellent health throughout the trial. Two sheep were euthanased and one was found dead throughout the trial. Each of these causes of death appeared unrelated to the administration of experimental vaccine. Necropsy reports for each of these cases can be found in Section 5.3.

2.2.3 Vaccine

2.2.3.1 Preparation

Vaccine was prepared in the Geelong laboratory as per the Standard Operating Procedure (SOP) in Section 5.2.1.

2.2.3.2 Administration

Vaccines were delivered subcutaneously alternating in either the left or right sides of the neck. An 18 gauge needle attached to a 3mL syringe was used for the delivery. At all times a member of the Study Personnel was present to direct the correct administration of vaccine to the correct study group.

2.2.4 Analysis of Study Results

Studies were first broken down to their constituent parts (eg. Study 1 groups only receiving 0.25mg/2mL, Study 3 0.25mg/2mL fresh vs. 6 months storage vs. 12 months storage). Groups were compared using the GraphPad Prism® analysis software. Group Means with error bars representing the standard error were used initially to point towards groups that may have significant differences. Further comparisons were generally made using the Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test to further elucidate any potential significant results seen with the initial test. The Kruskal-Wallis test is a non-parametric test which is suitable for the comparison of multiple groups. This test uses group medians in formulating its results. Scatter within groups was analysed using Standard Deviation, Standard Error of the Mean and/or the Coefficient of Variation.

2.2.5 Laboratory and Clinical Standards

2.2.5.1 Staff training

In most instances, project staff had existing expertise that was applied to the project eg. chemistry, animal handling skills and toxicology. In cases where researchers were given tasks outside of their normal expertise, "in-house" training was conducted to the satisfaction of the Project Leader that enabled those staff to reliably and competently perform the new duties under supervision.

2.2.5.2 Calibration:

Calibration of laboratory equipment such as pipettes was completed in-house at the AAHL.

2.2.5.3 Standard Operating Procedures:

Standard Operating Procedures can be found in Section 5.2.

2.2.5.4 Record Keeping

Procedural steps, observations (eg. injection site reactions), results (eg ELISA records) were all recorded on paper or electronically. Hard copy records were stored in AAHL lab books kept within the research areas at the AAHL. Most of the hard copies were subsequently electronically scanned or the data manually transferred into electronic spreadsheets.
All electronic records are to be stored in the CSIRO AAHL TRIM Records Management system (product of TOWER Software).

2.2.5.5 ELISA

All ELISA's were performed by Yu Cao and Agnieszka Michalewicz. The SOP's for the corynetoxin antibody ELISA and the antibody isotyping ELISA are found in Section 5.2.

2.2.6 Disposal of Study Materials

Sheep from the study were disposed of by high temperature rendering.

3 Trial Studies

3.1 Study 1

3.1.1 Experimental Design

- 3 vaccine doses, 5 vaccination regimes – 13 groups of 10 sheep
- Vaccine doses: 0.25, 0.5, 1.0 mg/2mL dose
- Sheep received 3 vaccinations plus an annual booster, across 5 vaccination regimes.
- Regime 1: Vaccines at 0, 2, 12 and 52 weeks.
- Regime 2: Vaccines at 0, 4, 12 and 52 weeks.
- Regime 3: Vaccines at 0, 6, 12 and 52 weeks.
- Regime 4: Vaccines at 0, 8, 12 and 52 weeks
- Regime 5: Group 9, a “gold standard” group was included which mimics the dose and regime that was used in the earlier successful challenge trials with this vaccine – vaccines at 0,9,24 and 52 weeks.

Table 3 Trial Design for Study 1

Group #	Regime #	Vaccine dose (mg/2mL dose)	1st vaccine (week)	2nd vaccine (week)	3rd vaccine (week)	Annual vaccine (week)
1	1	0.25	0	2	12	52
2	2	0.25	0	4	12	52
3	3	0.25	0	6	12	52
4	4	0.25	0	8	12	52
5	1	0.5	0	2	12	52
6	2	0.5	0	4	12	52
7	3	0.5	0	6	12	52
8	4	0.5	0	8	12	52
9	Gold Standard	0.5	0	9	24	52
10	1	1.0	0	2	12	52
11	2	1.0	0	4	12	52
12	3	1.0	0	6	12	52
13	4	1.0	0	8	12	52

3.1.2 Results

In Study 1 (as well as Studies 2 and 3) all groups tested produced a measurable corynetoxin antibody response which was significantly above baseline levels. This in itself is a good result for the vaccine. In the 1997 challenge study, where 9 out of 10 sheep survived a corynetoxin challenge, our estimate is that sheep that survived the challenge had antibody titres of around 300 at 3 – 5 weeks after their 3rd vaccination. These sheep had been vaccinated with the "gold standard" regime of 0, 9 and 24 weeks. They were challenged with corynetoxin slurry 2 weeks after the 24 week vaccination. The historical serum samples we have date from 1 week after this challenge (3 weeks after the 3rd vaccination). These antibody titres may provide some degree of comparison with the current study, in terms of magnitude of antibody response. Figure 1 shows a comparison of the 1997 results with the equivalent dose regime and time point in the current study. It must be

remembered that the 2009 data does not include a challenge, so care must be made with the comparison. It appears clear, however, that the titres obtained in the current study are at least as high and most likely higher, then those seen in the 1997 study.

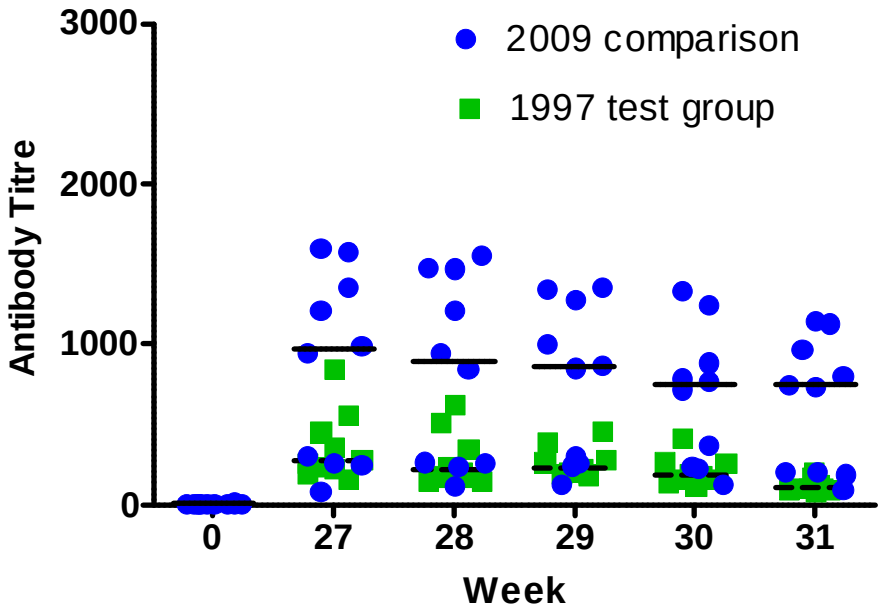


Figure 1. Comparison of antibody titre magnitude. 2009 comparison (Group 9) vs. the 1997 challenge study group. Dotted bars represent the mean antibody titre for the 1997 test group, with an overall mean titre value of 252. Solid bars represent the same for the 2009 comparison, with an overall mean titre of 752.

3.1.2.1 The optimum time for giving the booster injection of the ARGT vaccine after the first injection.

Immunologically it is accepted dogma that timing the second injection for just after the peak of antibody titre following the primary injection is expected to result in the best boost to antibody levels. The group mean antibody titres for regime 1 (vaccines at 0, 2, 12 weeks) are shown in Figure 2. It is obvious that by 2 weeks after the initial vaccine antibody levels have not peaked. Figure 3 shows the group mean antibody titres for regime 4. This perhaps allows a better perspective as to the antibody kinetics after the first vaccination. Antibody titres appear to reach a maximum by 5 – 6 weeks after the first vaccination. Based on this, regimes 3 and 4 seem the more immunologically

sound choice for a vaccination program. Regimes 3 and 4 (Figures 4 and 3) display an observed boost to antibody levels following the second vaccination. Using this reasoning regime 2 would not seem the rational choice either, where antibody levels are only approaching their peak by week 4 after vaccination, as Figure 5 displays.

The dose of vaccine (0.25mg/2mL, 0.5mg/2mL and 1.0mg/2mL) does not appear to influence when this peak occurs. Changing the vaccine volume to 1mL, as occurs in Study 2, does not appear to alter these antibody kinetics. This is discussed further in 3.2.

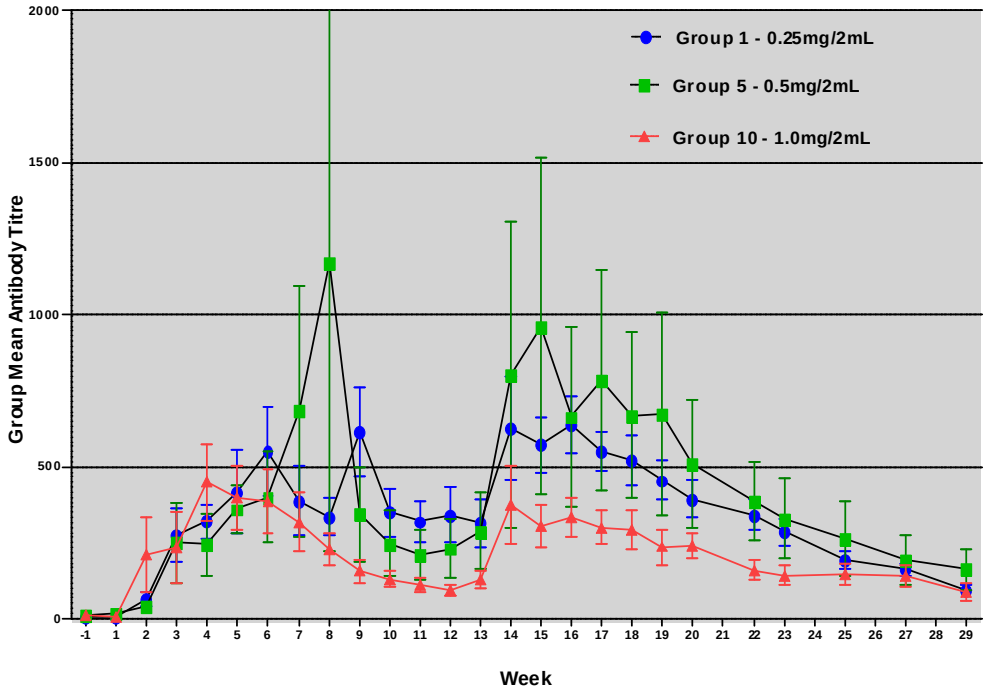


Figure 2. Antibody kinetics for regime 1. Vaccines were given at 0, 2 and 12 weeks of age. Error bars are +/- SEM. Titres are only just rising by the time of the first boost at 2 weeks.

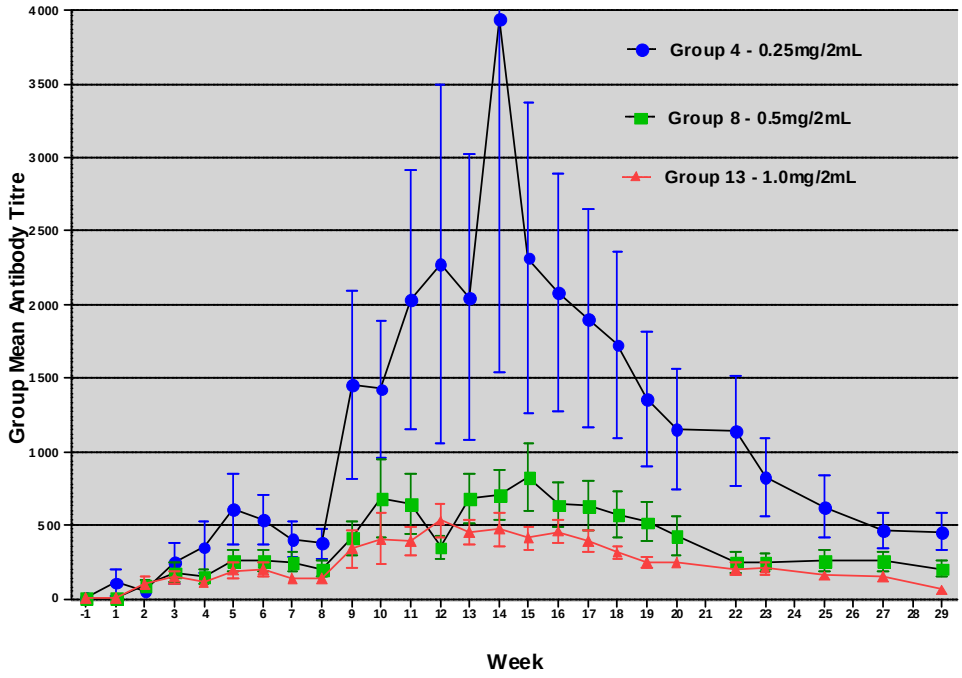


Figure 3. Antibody kinetics for regime 4. Vaccines were given at 0, 8 and 12 weeks. Antibody kinetics following the first vaccination shows an apparent maximal titre by 5-6 weeks after vaccination.

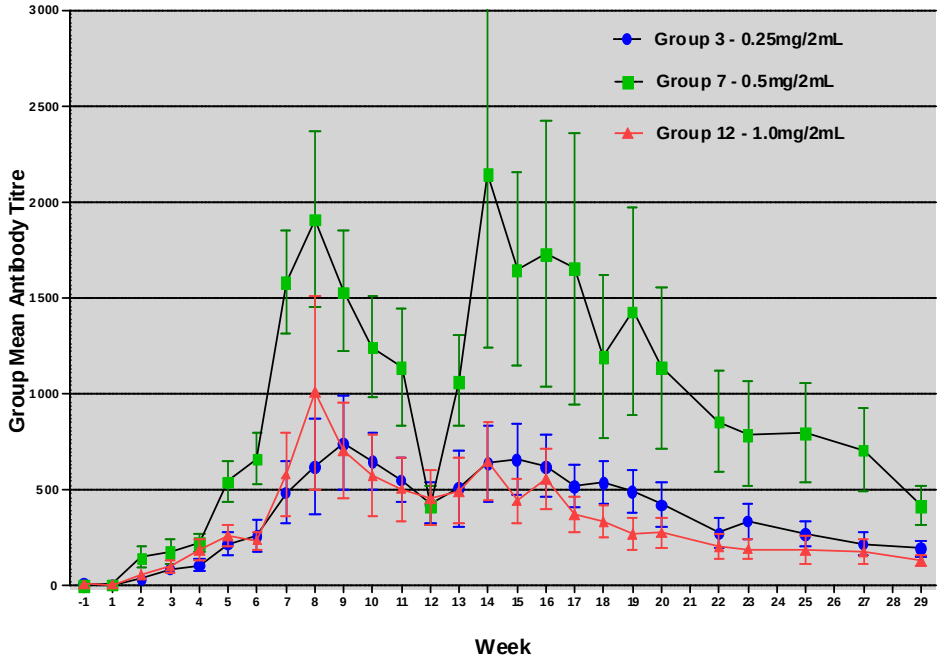


Figure 4. Antibody kinetics for regime 3. Vaccines were given at 0, 6 and 12 weeks. Error bars are +/- SEM. Vaccines were given at 0, 6 and 12 weeks. A number of groups seemed to have just reached their peak by 6 weeks.

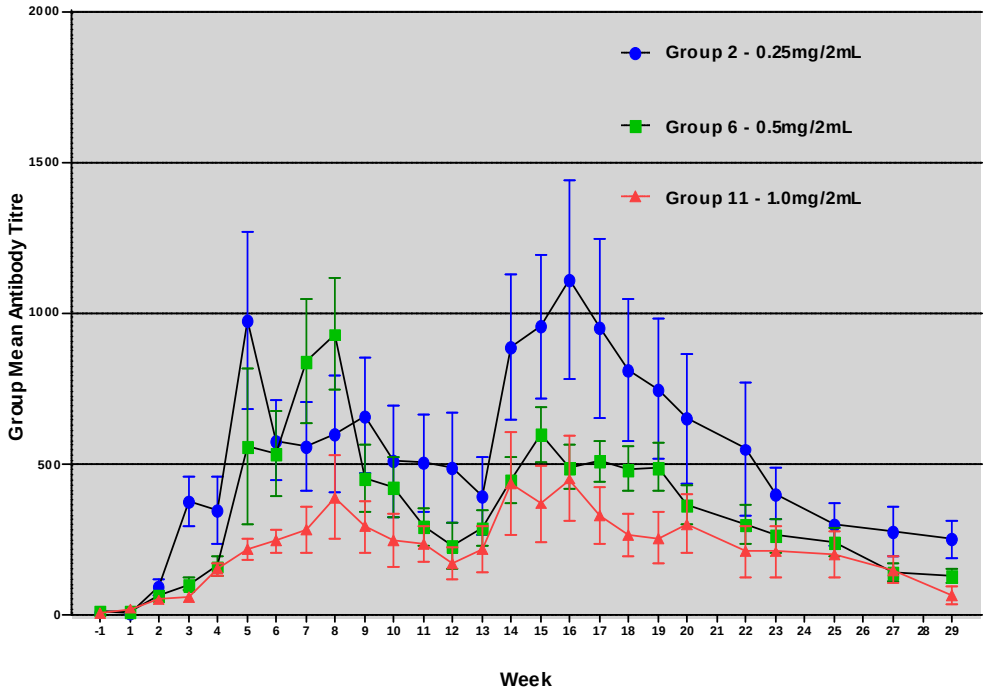


Figure 5. Antibody kinetics for regime 2. Vaccines were given at 0, 4 and 12 weeks. Error bars are +/- SEM. Group 2 appears to be reaching a maximal antibody titre to the first vaccination by approx. 4 weeks.

The distance between the first booster injection and the second booster injection should also be considered. With a first booster at 8 weeks (Fig. 3), antibody titres appear to have peaked and be just on the decline by the time of the second booster injection at 12 weeks. From an immunological point of view this is a good profile. With a first booster at 6 weeks (Fig. 4) it appears that titres have peaked and have been decreasing for nearly a month before the 12 week boost. While the means of regime 4 appear to get a bigger boost from the 12 wk vaccination compared to regime 3, this is not however seen when medians are compared.

Immunologically regime 4 appears to be reasonable to recommend. Regime 3 appears nearly as sound from an immunological point of view.

Is it wise to discount regimes 1 and 2 purely based on this immunological dogma? There would have to be some other justification in order to recommend regime 1 or 2, in particular regime 1. The range of antibody titres within a group, the actual level of measured antibody and the regime's

response to an annual vaccination could all be factors important in this decision. These will be looked at in following sections.

3.1.2.2 The optimum concentration of immunogenic conjugate required to stimulate the highest and most stable (persistent) level of corynetoxin antibodies i.e., the kinetics of the antibody production

Section 3.1.2.1 concluded that regimes 3 and 4 are probably the best places to start in terms of regimes. Figure 6 shows the first 30 weeks of monitoring for the 3 vaccine doses tested in regime 3.

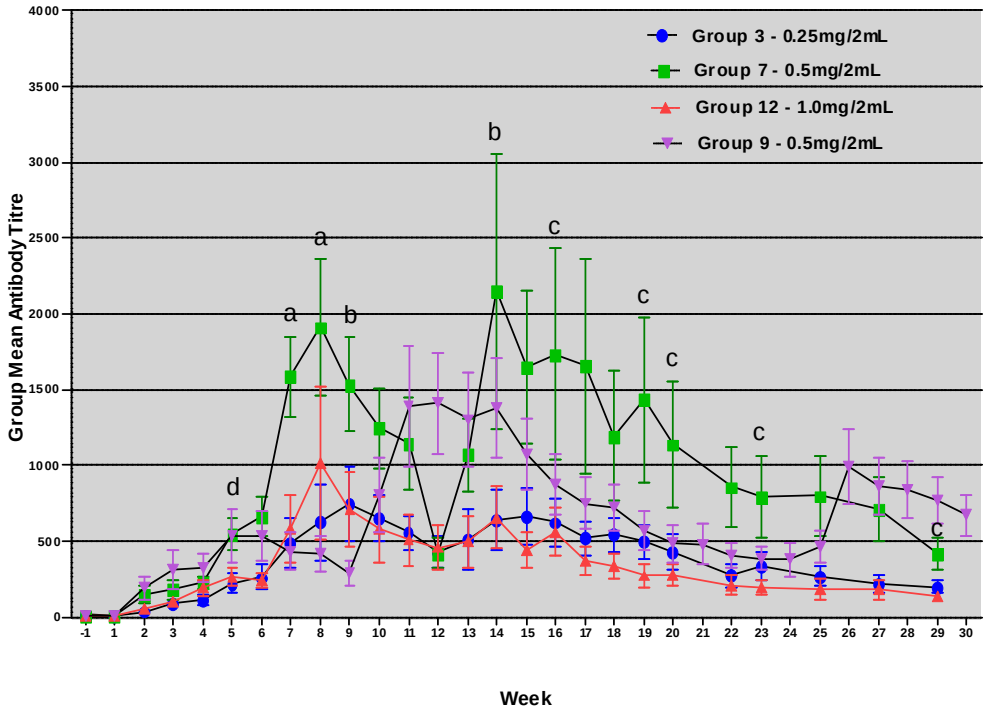


Figure 6. Group Mean Antibody titres for regime 3. Error bars are +/- SEM. Group 9 is included for comparison. 'a' denotes group 7 mean differs from groups 3 and 12, P<0.05. 'b' denotes means do not differ, P>0.05. 'c' denotes group 7 mean differs from 12, P<0.05. 'd' denotes means appear to differ, but further statistical tests unable to elucidate which groups differ.

The 0.5mg/2mL dose group appears to perform the best using this regime. There is some suggestion that this dose has performed significantly better up until the second vaccination. After the second and third vaccinations it seems to perform significantly better, with titres continually being better than the 1.0mg/2mL dose to the 28 week mark. Statistical analysis has not been

applied to every week. Significant or suspected interesting time points were chosen for analysis. An important consideration is the variability in antibody responses within a group. If the Coefficient of Variation (%CV) is used to try to quantify the scatter of titres between groups, there appears little difference between dose groups. Mean %CV were 85, 87 and 100 for groups 3, 7 and 9 respectively, across the 30 week period examined. The %CV gives us an idea as to the scatter as a proportion of the mean. The mean SD figures for the 0.5mg/2mL dose are 2 to 3 times higher than for the other dose groups. This appears a consistent pattern across the treatment groups for those vaccines groups that appear to produce superior mean/median antibody titres. It may just be a function of individual sheep variation to vaccines. ie. A sheep destined to respond poorly to a vaccine may continue to struggle even with a perhaps superior dose/formulation of vaccine, whereas those sheep who respond better to the vaccine will respond more readily to a superior dose of vaccine.

Figure 7 shows the first 30 weeks of monitoring for the 3 vaccine doses of regime 4. Using this regime the 0.25mg/2mL dose group at first glance seems to provide good results. The scatter among antibody responses from this dose group however is quite large, certainly overlapping all other groups when SD is compared. The overall SD for the first 30 weeks is between 5 and 10 times higher for the 0.25mg/2mL dose group than the other dose groups in this regime. The %CV is similar for groups 8, 9 and 13 while for group 4 it is 25% larger. There is some statistical significance to the higher results from the 0.25mg/2mL dose group; however in the main it would be hard to conclude a significant difference between the 0.25 and 0.5mg/2mL dose groups, at least. Comparing the group medians, as in Figure 8, provides another perspective, removing the large peaks seen with the means. Within the group of 10 sheep in group 4, there were 2 sheep, 1 in particular, that produced antibodies at levels 5 – 10 times that of other reasonable responders. This does skew the mean results; however the statistical analysis used is based on the group medians so these large results are accounted for.

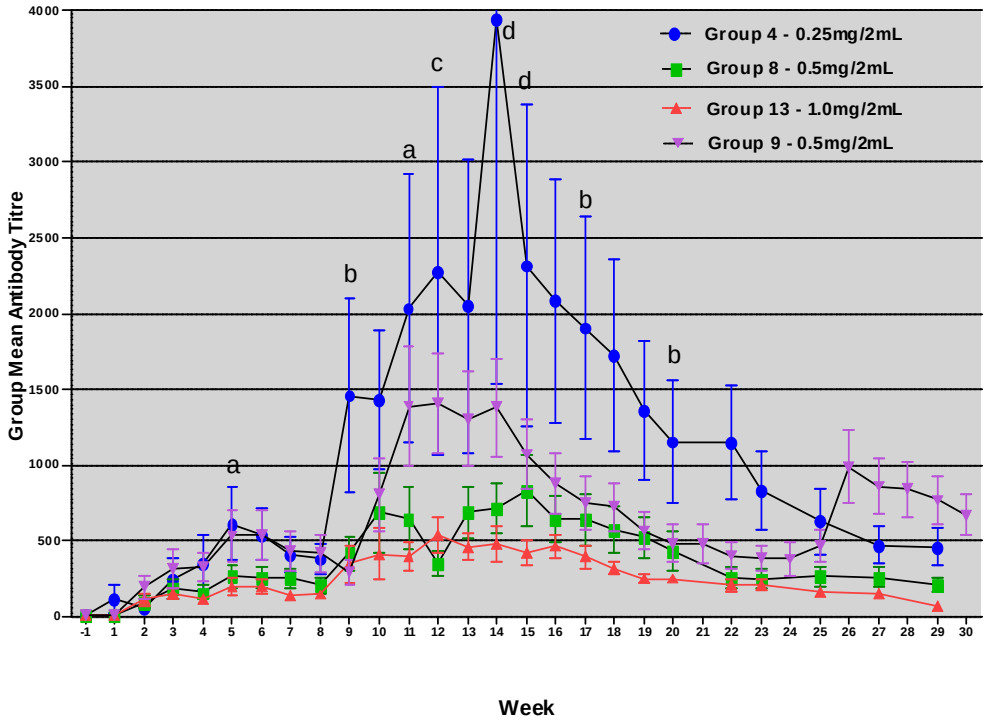


Figure 7. Group Mean Antibody titres for regime 4. Error bars are +/- SEM. Group 9 is included for comparison. 'a' denotes no significance between groups 8 and 9. 'b' denotes means do not differ, P>0.05. 'c' denotes means of groups 4 and 8 differ, P<0.05. 'd' denotes means of groups 4 and 13 differ, P<0.05

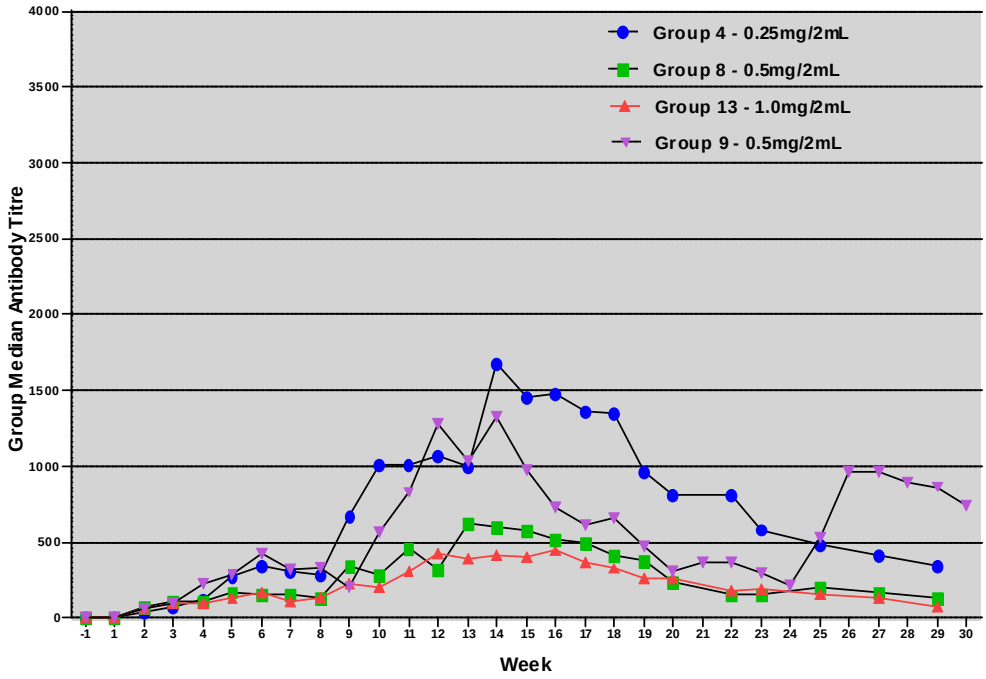


Figure 8. Group medians for regime 4. Group 9 is included for comparison.

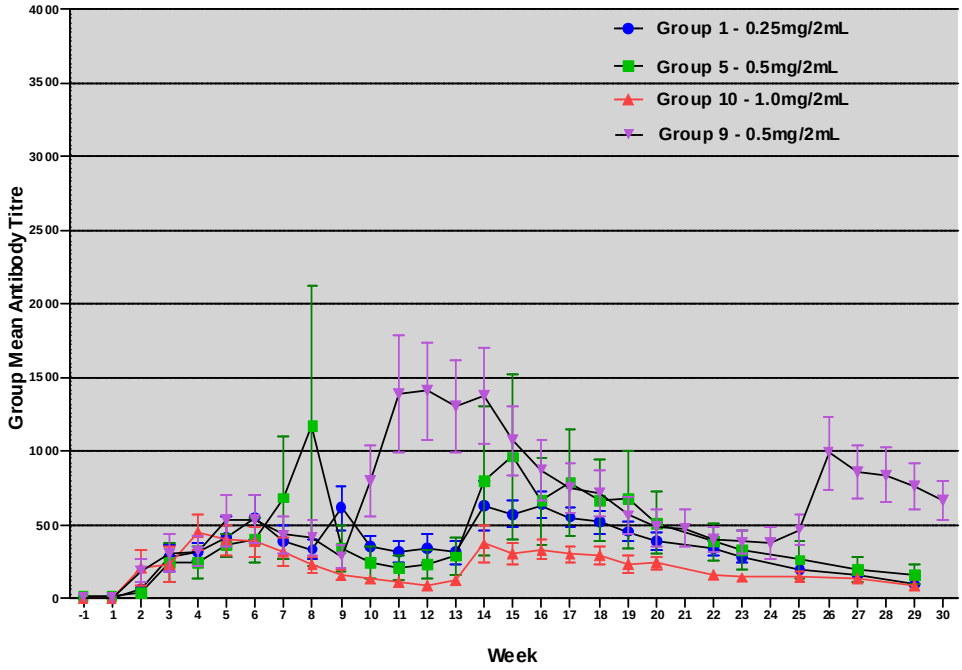


Figure 9. Group mean antibody titres for regime 1. Error bars are +/- SEM. Group 9 is included for comparison.

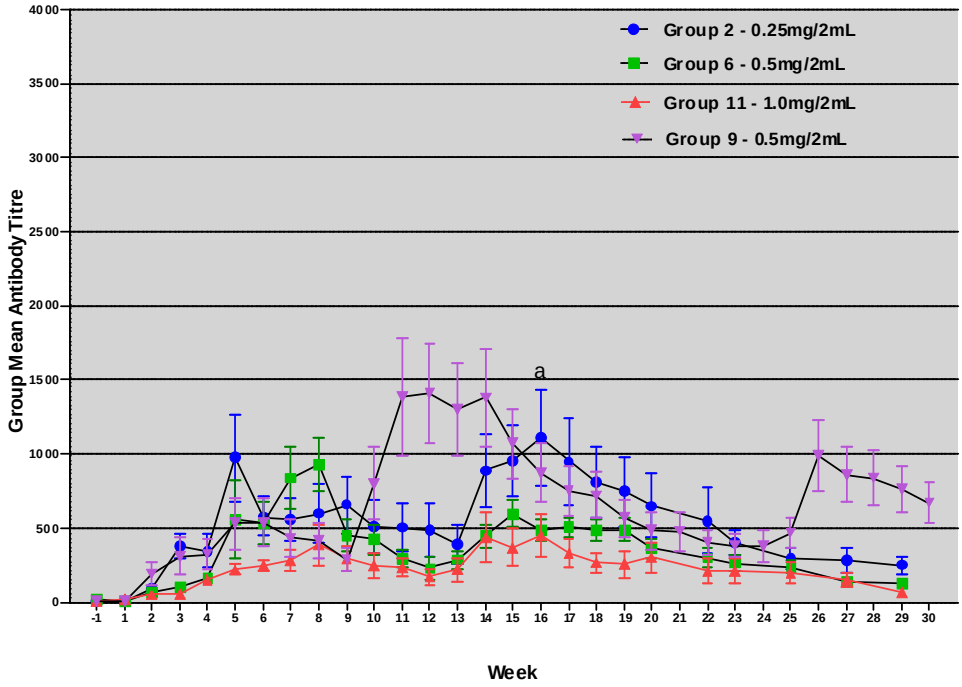


Figure 10. Group mean antibody titres for regime 2. Error bars are +/- SEM. Group 9 is included for comparison. 'a' denotes no significance between groups at week 16, P>0.05.

Figure 9 shows group means for regime 1. There appears a trend towards group 5 (0.5mg/2mL) providing best results with this regime. Certainly there is no statistical significance to the trend. All dose groups performed similarly with this regime. All groups performed similarly with regime 2 (Figure 10), with a trend towards group 2 being the best with this regime. No statistical significance was detected between groups 2, 6 and 11.

Memory effect after the annual vaccination

All groups produced a significant antibody response when vaccinated at 52 weeks after the first vaccination. In general, responses were at least equivalent to those obtained with the gold standard regime. Antibody titres generated after the annual vaccination were in general higher than those generated after the primary vaccination and the responses occurred quicker than with the primary vaccination, allowing us to conclude that there was a significant memory effect to the antibody responses. Maximal titres to the annual vaccination were obtained after only two to three weeks. After the primary vaccination (0 weeks) maximal titres were obtained after 4-7 weeks. The rate of decline of titres following the annual vaccination appears similar to that obtained after the primary vaccination. Due to the study design all sheep that received a primary vaccination received a second vaccine by week 9, which means a full picture of decline following 1 vaccination is hard to obtain. Figure 11 shows the results of an annual vaccination with sheep vaccinated with regime 1. There were no significant differences in group mean titres with this regime. Similar results were obtained with regime 2 (Figure 12).

For regime 3 (Figure 13), the 0.5mg/2mL group 7 seems to respond better in terms of antibody production after the annual vaccination. This is generally a trend. Means of group 7 were not statistically different from those of the gold standard group and only marginally better than the 0.25mg/2mL group 3 at 1 time point. At a number of time points it stimulated antibody levels statistically higher than those from group 12 (1.0mg/2mL).

The better profile observed after annual vaccination with group 4 using regime 4 is a trend only (Figure 14). No statistical significance was detected. P

values were between 0.06 and 0.07 for weeks 2, 4, 6 and 8, suggesting that the difference between means was close to being statistically significant.

Other than trends to the 0.5mg/2mL dose group with regime 3 and the 0.25mg/2mL dose group with regime 4, all groups appear to have performed similarly at the annual vaccination. Titres stimulated were at least equivalent to those titres that were present in sheep that survived challenge in the 1997 study. As such, there is no information in the annual vaccination results that detracts from regimes and doses that performed well in the initial three vaccination course.

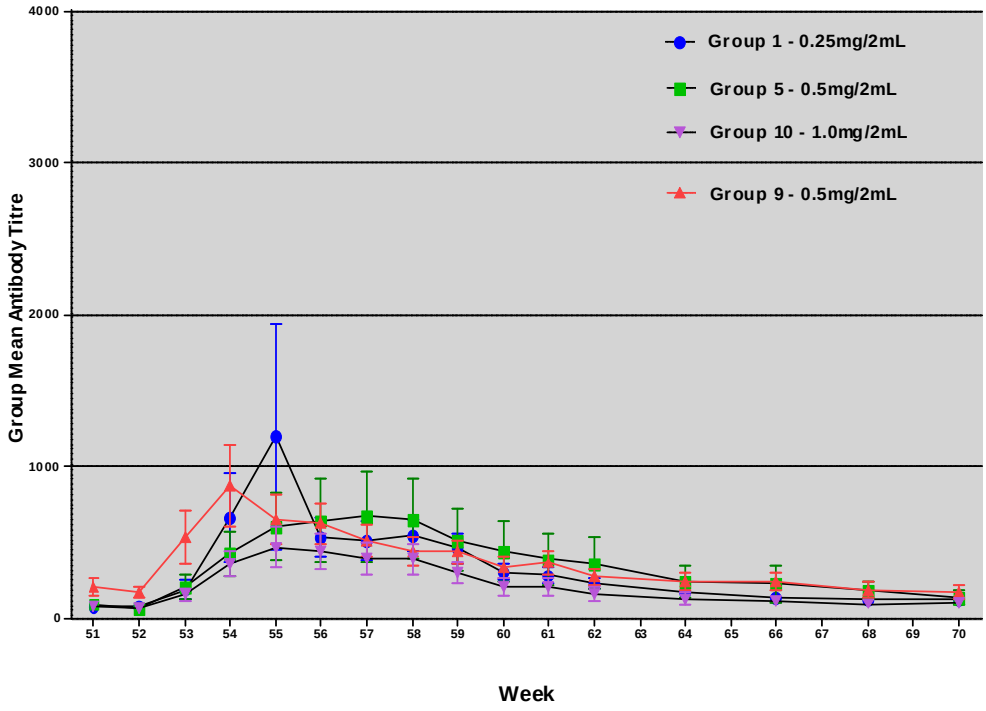


Figure 11. Regime 1 antibody kinetics after annual vaccination at 52 weeks. Error bars are +/- SEM. Group 9 is included for comparison.

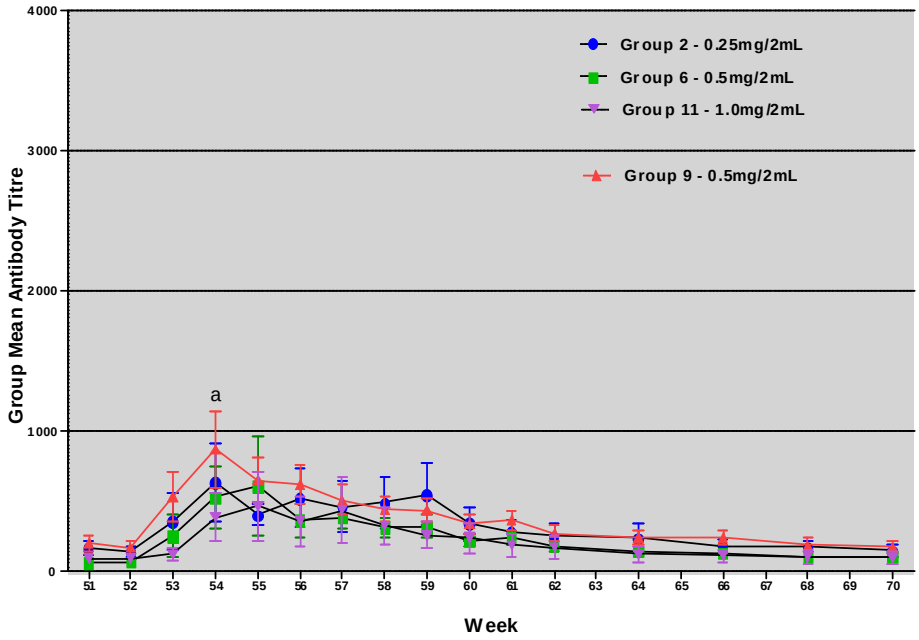


Figure 12. Regime 2 antibody kinetics after annual vaccination at 52 weeks. Error bars are +/- SEM. Group 9 is included for comparison. 'a' denotes group means do not differ at week 2, P>0.05.

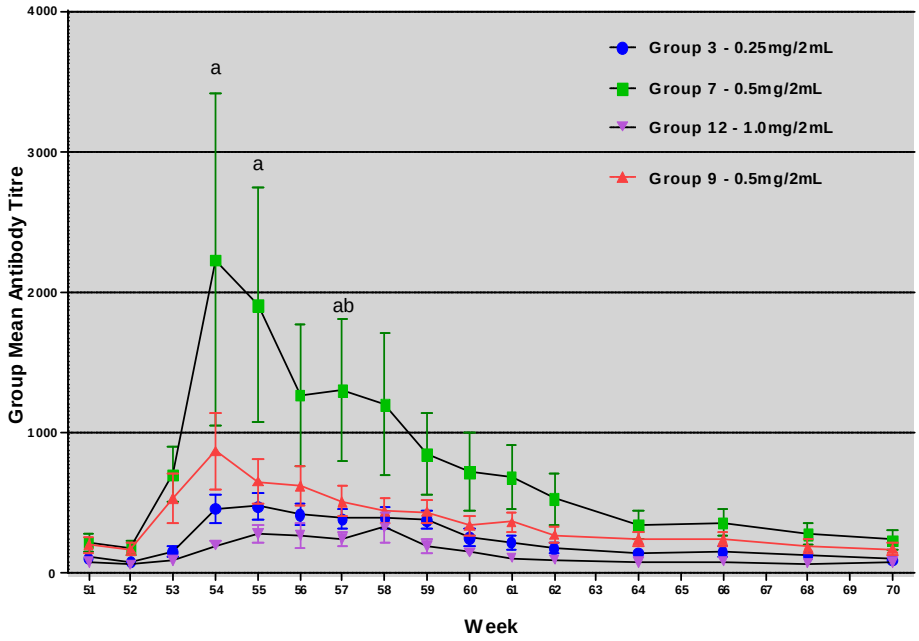


Figure 13. Regime 3 antibody kinetics after annual vaccination at 52 weeks. Error bars are +/- SEM. Group 9 is included for comparison. 'a' denotes group means differ between groups 7 and 12, P<0.05. 'b' denotes significant differences between groups 7 and 3, P<0.05

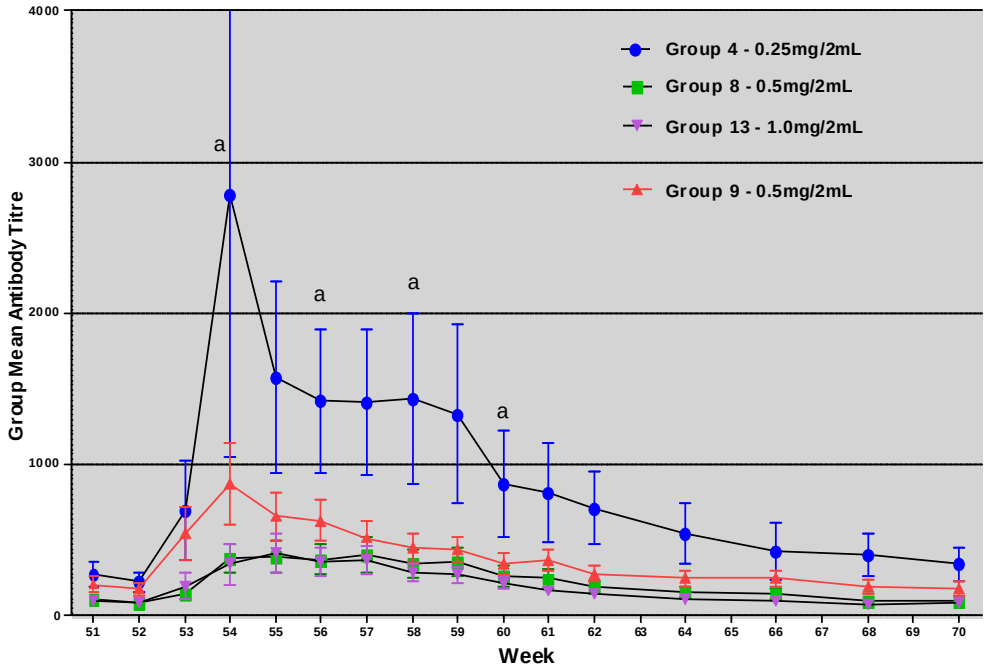


Figure 14. Regime 4 antibody kinetics after annual vaccination at 52 weeks. Error bars are +/- SEM. Group 9 is included for comparison. 'a' denotes statistical analysis was carried out on these time points and no significance was detected, P>0.05.

3.1.2.3 Antibody isotypes induced

The corynetoxin antibody ELISA will detect both Immunoglobulin A (IgA) and Immunoglobulin M (IgM) antibodies stimulated by the vaccine. IgM antibodies are typically the first induced to a vaccine, usually reaching a peak and returning to baseline levels within approximately three weeks of vaccination. IgG antibodies are slower to induce after initial vaccination, but are desirable in a vaccine response as they are thought to be responsible for the long-lasting effects of vaccination (the memory response) and have a high affinity for their antigen. The antibody kinetics displayed in the results of this trial suggests that the antibody isotypes induced follow this classic pattern, with antibody titres generally rising after 3 – 4 weeks. In addition, selected groups were chosen to confirm these antibody isotypes with an antibody isotype ELISA. These isotype-specific ELISA results confirmed that, at critical time points after vaccination, IgG isotypes were present (Figure 15) and that, as expected, induced IgM antibodies were only present for two to three weeks after the primary vaccinations (data not shown). Figure 15 gives a broad

overview of the IgG isotypes induced. The red lines represent the normal group mean antibody titres. The green lines represent IgG1 and 2 ELISA results. These 2 ELISA's are not calibrated to each other, so please disregard the actual titre values. What is important is that the IgG1/2 kinetics is similar to the group mean antibody kinetics. This, combined with the time course of induction of the corynetoxin antibody titres leads us to conclude that antibody isotypes induced by the vaccine follow the desirable profile described above.

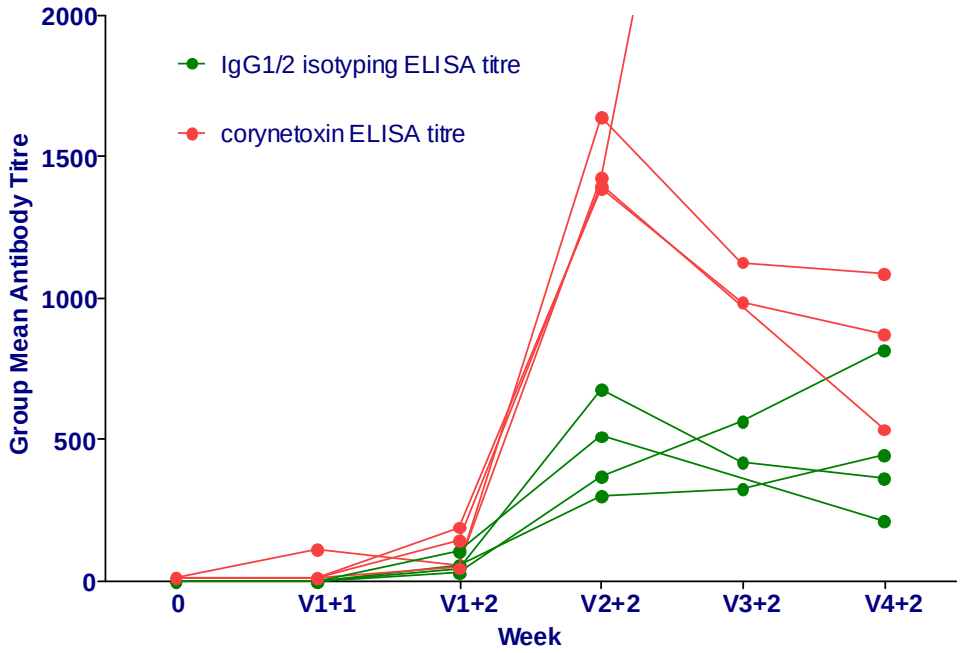


Figure 15. Isotype specific IgG1/2 kinetics compared to the standard ELISA antibody kinetics. Studies 1 and 2 are represented. Each line represents a group mean. V1 represents the first vaccine, V2 represents the first booster, V3 represents the second booster, V4 represents the annual vaccination.

3.2 Study 2

3.2.1 Experimental Design

- Vaccine volume and booster requirements – 2 groups of 10 sheep each
- Vaccine dose of 0.25mg/1mL (compared with a 2mL volume previously used)
- 2 vs. 3 injection regime

Table 4 Trial Design for Study 2

Group #	1st vaccination (week)	2nd vaccination (week)	3rd vaccination (week)	Annual vaccination (week)
14	0	9	24	52
15	0	9	-	52

3.2.2 Results

3.2.2.1 Which of two volumes of vaccine formulation produces the better antibody titre kinetics and the best memory effect after 12 months?

A vaccine volume of 1mL appears to produce results similar to a 2mL vaccine volume in terms of antibody levels stimulated and the kinetics of the response (Figure 15). The 1mL vaccine volume also produced a similar memory response after annual vaccination (Figure 16).

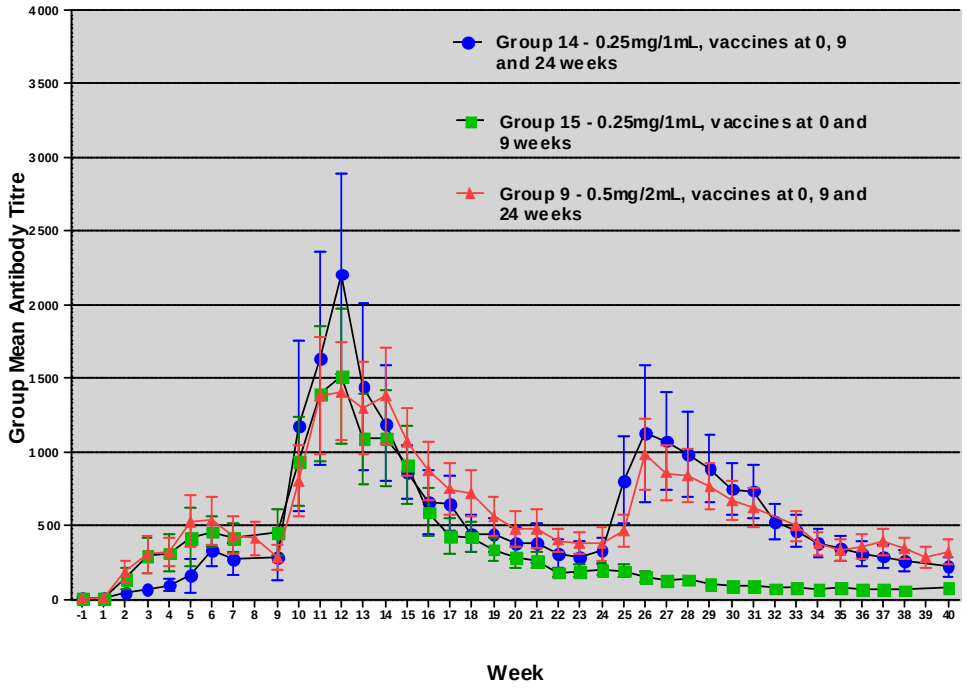


Figure 16. Study 2. Comparison of 1mL (groups 14, 15) vs. 2mL (group 9) vaccine volume. Groups 14 and 9 were vaccinated at 0, 9 and 24 weeks. Group 15 was vaccinated at 0 and 9 weeks.

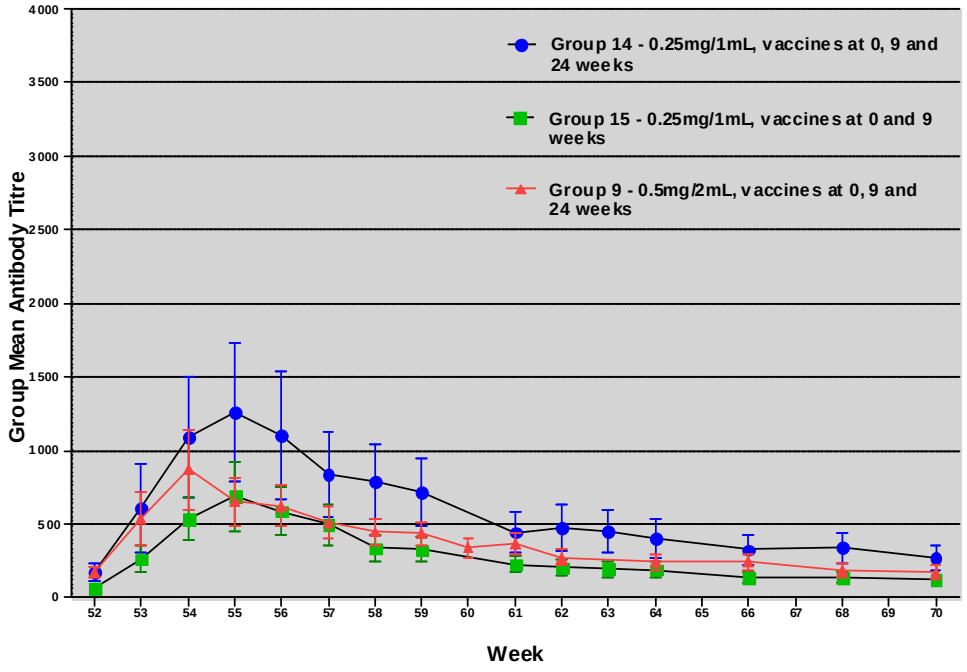


Figure 17. Study 2 responses to annual vaccination at 52 weeks. Error bars are +/- SEM. Statistical analysis on weeks 1 – 6 found means did not differ, P>0.05.

3.2.2.2 Whether a secondary booster injection is required

In terms of the memory response to an annual vaccination, results were similar when an initial 2 vaccine regime was compared to a 3 vaccine regime (Figure 16). This is important as one of the concerns of a 2 vs. 3 vaccine regime is that the 2 vaccine regime may not be able to produce similar memory antibody response after an annual vaccination. Our results show that this is not the case.

In terms of antibody titres achieved and the kinetics of production, the 2 vaccine regime appears promising. Further suitability of a 2 vs. 3 regime would be determined with challenge trials.

3.2.2.3 Which antibody isotypes are produced? Does the IgG get re-induced after 12 months or is it a re-induction of IgM mainly?

Antibody isotypes induced with both the 1mL vaccine volume and the 2 vaccine regime appear similar to those described in 3.1.2.3. They follow the classical IgG response, both at the initial vaccine regime and at the annual vaccination time point.

3.3 Study 3

3.3.1 Experimental Design

- Investigate the stability of the vaccines stored for 6 and 12 months (6 groups of 10 sheep each)
- Vaccine doses of 0.25, 0.5, 1.0 mg/2mL dose
- Vaccines stored at 4^{o}C for 6 and 12 months prior to vaccinations commencing

Table 5 Trial Design for Study 3

Group #	Length of vaccine storage (months)	Vaccine dose (mg/2mL dose	1st vaccination (week)	2nd vaccination (week)	3rd vaccination (week)
16	6	0.25	0	8	12
17	6	0.5	0	8	12
18	6	1.0	0	8	12
19	12	0.25	0	8	12
20	12	0.5	0	8	12
21	12	1.0	0	8	12

3.3.2 Results

3.3.2.1 Do the 3 immunogenic conjugate levels retain their activity when stored for 6 months and 12 months?

<u>0.25mg/2mL vaccine dose (Figure 17)</u>

This dose of vaccine appears to have maintained its antibody generating activity with both 6 and 12 months of storage at 4^0C. First glance at the graphical data appears as if there has been a decrease in antibody generating activity with storage of this dose, but once the Standard Error is calculated it becomes obvious that any difference is not significant. Kruskal Wallis statistical analysis on suspected interesting time points (based on standard errors not overlapping) suggests there is a low probability that any variation is due to any drop in vaccine potency with time in storage.

<u>0.5mg/2mL and 1.0mg/2mL vaccine doses (Figures 18 and 19)</u>

These doses of vaccine have maintained their abilities to elicit a corynetoxin antibody response with 6 and 12 months of 4^0C storage.

At several of the time points the stored vaccine (particularly the 6 months stored vaccine) has produced higher antibody levels compared to the fresh

vaccine. In many cases these higher values are statistically higher. We can only postulate as to why this may be the case. Biological variation between different generations and mobs of sheep is the main reason why a result such as this is possible. The objective of including these groups of sheep was to test whether these doses of vaccine retain their activity after 6 and 12 months of storage. We can conclude that they have retained their activity. Importantly, after storage all vaccines were able to stimulate antibody levels to the level above which we believe to be protective, based on previous challenge studies.

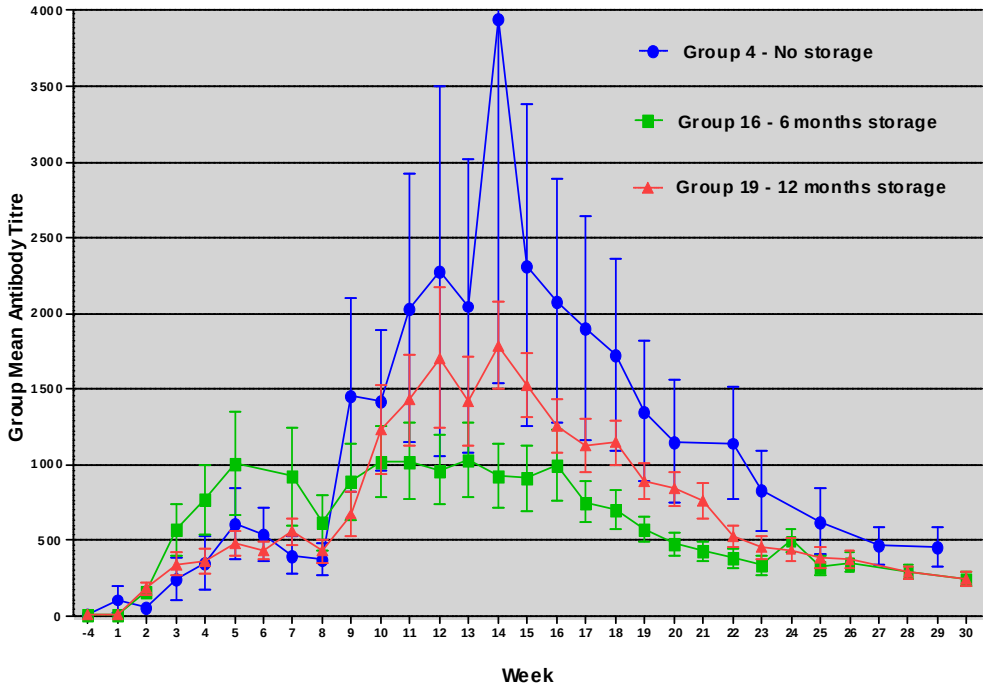


Figure 18. Storage stability for 0.25mg/2mL vaccine dose. Error bars are +/- SEM. All groups received vaccinations at 0, 8 and 12 weeks. Statistical analysis was completed on weeks 1, 9, 14, 15, 17, 18, 19, 20, 22. Means were found not to differ, P>0.05.

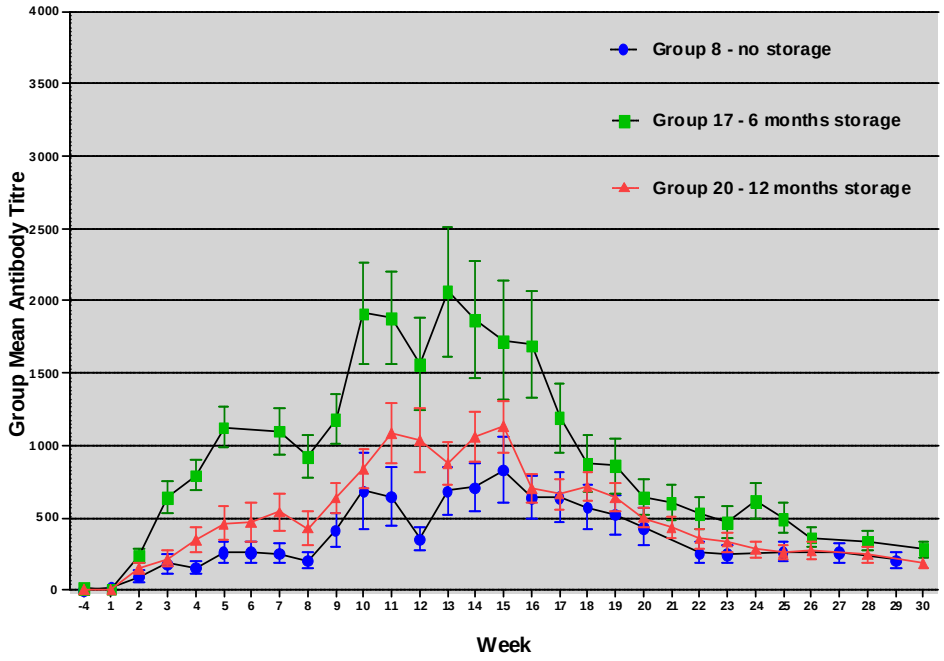


Figure 19. Storage stability for 0.5mg/2mL vaccine dose. Error bars are +/- SEM. All groups received vaccinations at 0, 8 and 12 weeks. Analysis of week 5 found group 17 had groups means significantly higher than groups 8 and 20, P<0.05. Analysis of week 10 found a difference between groups 17 and 8, P<0.05.

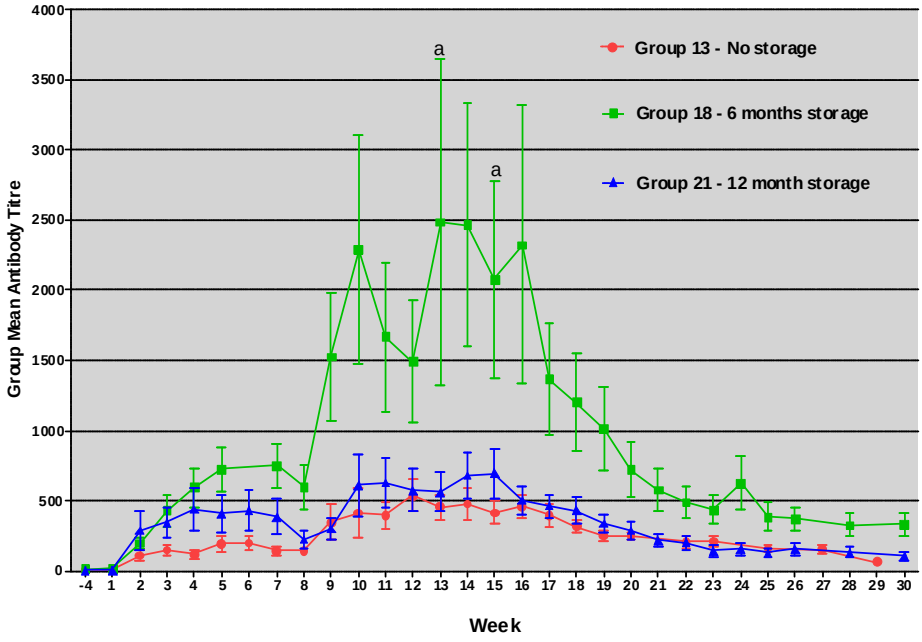


Figure 20. Storage stability for 1.0mg/2mL vaccine dose. Error bars are +/- SEM. All groups received vaccinations at 0, 8 and 12 weeks. 'a' denotes mean of groups 13 and 18 differ at these weeks, P<0.05. No difference in groups 18 and 21 at these weeks.

3.4 Injection site reactions

The vaccine was easy to inject subcutaneously with an 18 gauge needle, with little resistance to depression of the syringe plunger. Sheep did not display any adverse reaction to the vaccine, other than would be naturally expected due to handling and the piercing of the skin with the needle. There was no lethargy noted in the days after vaccine injection.

Injection site swelling is to be expected with any vaccine, this is the normal process of generating an immune response. The concern for a commercial vaccine is whether the injection site reactions persist. Out of the 210 sheep vaccinated in this project, 6 sheep had small injection site reactions that persisted to slaughter. The details of these reactions are shown in Table 6. The rate of injection site reactions to this vaccine is well within industry limits.

Table 6 Injection site reactions persisting to slaughter.

Study #	Group #	Number of sheep	Size of injection site reaction (mm diameter)
1	5	1	5
1	9	1	10
1	12	1	15
2	14	1	5
3a	18	1	20
3b	19	1	15

4 Study Conclusions

The vaccine has been robust in performance. There seems a high degree of tolerance to differing doses, timing and storage of the vaccine. All groups responded well to an annual vaccination. There was little significant difference in antibody kinetics among the groups and titres produced were above what is thought to be protective based on previous successful challenge studies.

4.1 Vaccine Regime

4.1.1 Three vaccine regimes

A second vaccination at 6 – 8 weeks followed by a third at 12 weeks appears the most immunologically sound choice. At these time points antibody titres from the previous vaccine had recently past their peak. These antibody kinetics are expected to result in the best boost to antibody levels. This was observed in this study, with better second and third vaccine boosts to antibody levels observed with regimes 3 and 4 compared to 1 and 2. To add weight to this recommendation, regime 4 was tested in Study 3, with consistent results.

The gold standard regime of 0, 9 and 24 weeks performed consistently in both Study 1 and 2 in terms of antibody titres produced. In terms of real world practicality, the gold standard regime may be difficult to manage if indeed protection is only obtained after the 24 week third vaccination. An essential third vaccination would be impossible to give to lambs before their first ARGT season. It may well be, given the reasonable titres produced with a 2 vaccine regime in Study 2, that protection is possible after the second vaccination at week 9. The antibody kinetics of the gold standard regime seems to indicate that the third vaccination does not produce a boost in antibody levels above those of the second vaccination. This increase over the preceding vaccines antibody levels is classically the reason for booster vaccines. (Providing a boost in antibody levels above and beyond that stimulated by the previous vaccine)

4.1.2 Two vaccine regimes

In this project, perhaps the most important parameter measured that allows for a comparison between 2 and 3 vaccine regimes is the response to an annual vaccination. The concern is that a 2 vaccine regime would not be enough to elicit a strong memory response to an annual vaccination. This project found no difference in terms of antibody kinetics between a 2 and 3 vaccine regime after the annual vaccination. This finding, coupled with consistent titres obtained after the initial 2 dose regime, are positive. They are enough to recommend a 2 vaccine regime be considered for future challenge trials. The benefits of a 2 vs. 3 regime for the farmer are obvious.

4.2 Vaccine Dose

The 0.25mg dose was chosen, based on results in Study 1, for both Studies 2 and 3. It has performed consistently in all three studies, in particular when linked to regime 4 and the gold standard regime. This consistency adds weight to recommending this dose for future challenge studies.
The 0.5mg dose has also performed equally consistently across the studies and can also be recommended. Given that the 0.25mg dose appears to have produced antibody profiles at least equal with the 0.50mg dose, there is a reasonable case to be made for trialling a dose lower than 0.25mg.
While the 1.0mg dose seems to consistently trend to stimulate lower antibody titres, often these titres are still within the range that we believe to be protective against corynetoxin challenge. When compared statistically, often there are no statistical differences between the 1.0mg dose and the other two doses. Given the trend towards lower titres with the 1.0mg vaccine dose, it is, however, hard to recommend this dose over the two lower doses. The trending towards lower titres perhaps should alert us that there may be some impediment to immunity with the higher doses. In all likelihood this would not be the case. More likely it is just biological variation that is responsible for these lower titres.

4.3 Vaccine Volume

Our results demonstrate clearly that in terms of antibody kinetics there is no difference between a 1mL and a 2mL vaccine volume.
There are certainly advantages to using a smaller vaccine volume in terms of decreasing the costs of production (adjuvant costs, inclusion in multi-disease vaccines are examples). Farmers are however accustomed to vaccines that are up to 2mL in volume (many Clostridial vaccines use a 2mL volume), so this would not be an issue for sheep farmers.

4.4 Vaccine Stability

Storage of all doses of vaccine for 6 and 12 months at 4^0C did not reduce their activity. Activity was measured in terms of corynetoxin antibody kinetics in comparison to the freshly prepared vaccine.

4.5 Vaccine safety

4.5.1 Vaccine tolerance

The vaccine was easy to inject subcutaneously with an 18 gauge needle, with little resistance to depression of the syringe plunger. Sheep did not display any adverse reaction to the vaccine, other than would be naturally expected due to handling and the piercing of the skin with the needle. There was no lethargy noted in the days after vaccine injection.

4.5.2 Injection site reactions

The rate of injection site reactions to the vaccine was low and certainly well within the acceptable limit required of a commercial vaccine. The accepted cut-off point for the rate of injection site reactions seems to be 5%. Out of the 210 sheep vaccinated in the study, less than 3% injection site reactions persisting to slaughter. Persisting injection site reactions were very minor in nature and would have had no clinical effect on the health of the sheep.

4.6 Antibody kinetics

As expected for outbred animals, there was significant variability within a group of sheep in terms of the magnitude of the measured corynetoxin antibody response. In general terms, however, the vast majority of sheep vaccinated in this study produced antibody levels that would be expected to be protective against a corynetoxin challenge. A degree of biological variation is to be accepted with any vaccine.

The pattern of induced antibody levels appears consistent with what is seen with other commercial vaccines. The stimulated antibodies appear predominantly IgG in nature.

4.7 Recommendations

The vaccine was well tolerated by the sheep, with injection site reactions well within industry standards. The vaccines appear stable for at least 12 months at 4^0C.

For future challenge studies we recommend that:

- 0.25mg/1mL be included as the vaccine dose
- Regime 4 be used, with vaccines at 0, 8 and 12 weeks
- A 2 vaccination regime is included in the studies.

There appears sufficient flexibility in the results obtained that a first boost between 6 and 9 weeks appears feasible. The 0.5mg vaccine dose has performed similarly to the 0.25mg vaccine dose.

5 Appendices

5.1 CSIRO AAHL Animal Ethics Committee Protocol

AEC1

APPLICATION FOR NEW PROJECT TO USE ANIMALS

CSIRO Livestock Industries
Australian Animal Health Laboratory
Animal Ethics Committee

CONTENTS

PROJECT DETAILS		
1.1	**Project Title**	
Development of a Vaccine Against Annual Ryegrass Toxicity		
1.2	**Investigator**	
Steven M. Colegate		
1.3	**Delegate in absence of Investigator**	
Agnieszka Michalewicz		

AEC1

EXPLANATION OF PROJECT IN PLAIN ENGLISH

2.1 Objective(s) of the project:

Briefly (½ A4 page maximum) explain the objectives of the project and expected benefits (in a way that can be understood by people without a scientific background)

This project is a data collection study of a proven experimental ARGT vaccine. It is necessary to define several parameters of commercial significance to make further development and marketing of the vaccine possible. Research objectives include:

- Finding the most favourable time for giving the booster injection after priming,
- Determining the optimum concentration of immunogenic conjugate necessary to stimulate the highest and most stable level of antibodies,
- Establishing if the secondary booster is required,
- Confirming and proving the most effective volume of vaccine formulation to produce the highest and the longest-lasting antibodies,
- Deciding which out of three tested levels of immunogenic conjugate provides the most stable vaccine when stored for 6 and 12 months

Basically, sheep will be maintained in paddocks and will be vaccinated with the experimental ARGT vaccine. Sera from sheep will be collected and assessed for corynetoxin antibody titres. The project aims to define the kinetics of corynetoxin antibody production and decline following vaccination.

2.2 Justification for the use of animals for research purposes:

Briefly (½ A4 page maximum) explain why the research cannot be carried out without the use of animals (in a way that can be understood by people without a scientific background)

It is not possible to determine the parameters listed in 2.1 without the use of the experimental animals. Vaccine effectiveness can only be proven if sera from vaccinated animals are collected and assessed at the appropriate time indicated in section 11 of the application. The sheep will not suffer during the experiment. Vaccination and bleeding procedures will cause minimal stress to the animals.

APPLICATION DETAILS

3.1 Protocol Number:

1186

3.2 File: 98/243-28 | **Meeting 2006/4**

3.3. Date of submission:

29.09.06

DATES

4.1 Expected commencement date:

15.01.07

4.2 Expected completion date:

1.10.09

PROJECT BACKGROUND

5.1 Project code:

kw44b

5.2	**Agent/s to be used:**
	modified Tunicamycin-Australian FCS conjugated vaccine
5.3	**Has this experiment been previously conducted elsewhere?** Type ***'Yes'*** or ***'No'***.
	Yes
5.4	**Reference (if applicable)**
	Australian Patent application No 66010/98, "Development of a Vaccine Against Annual Ryegrass Toxicity", Proceedings Int. Symps. Poisonous Plants 1998 PGS, 165-168 authors Than, K..A., Cao, Y., Michalewicz, A., Edgar, J.A., Cab. Int.
5.5	**If you answered *'Yes'* to item 5.3 then please explain why the work is being repeated:**

Future commercialisation requires optimisation of the formulation and dose regime, confirmation of raw material sources, establishing stability of vaccine.

AQIS AND OGTR

6.1	**Is the proposed experiment under the AQIS Premise System?**
No	
6.2	**Is the proposed experiment under the Office of the Gene Technology Regulator Guidelines (OGTR)?**
	No
6.3	**Are animals that have been used in a previous experiment to be used in this project?** If you answered ***'No'***, go to item 7.1 If you answered ***'Yes'*** complete items 6.4 and 6.5
	No
6.4	**What was the previous AEC1 number?**
6.5	**Outline what experimental procedures the animals proposed for re-use have already received. (refer to Australian code of practice for the care and use of animals for scientific purposes 2004 sections 3.3.11 and 3.3.12)**

AEC1

ANIMALS REQUIRED

7.1	**Species:** Scientific name (optional)	Ovis aries		
7.2	**Species:** Common name	Sheep		
7.3	**Strain:**	Merino-cross		
7.4	**Number required:**	200		
7.5	**Age:**	weaned lambs		
7.6	**Sex:**	wethers		
7.7	**Source of Animals:**	Victorian farm		

ANIMAL ACCOMMODATION

8.1	**Secure Laboratory Animal Suite**	
	Number of animals per cage/pen/box:	
8.2	**Secure Large Animal Facility**	
	Number of animals per cage/pen/box	
8.3	**Small Animal Facility: Level 6**	
	Number of isolators required:	
	Number of birds per isolator:	
8.4	**Small Animal Facility: Level 6**	
	Number of cabinets required:	
	Number of animals per cage/cabinet:	
8.5	**Werribee Animal Facility**	
	PC1 number of animals per pen:	
	PC2 number of animals per pen:	
	Non secure:	
	Paddock:	Yes
	Other accommodation requirements:	

INVESTIGATORS AND STAFF THAT WILL BE HANDLING THE ANIMALS

9.1	Name	Species	No. of Years Experience with this species	Procedure	Please explain why this person is suitable to perform this procedure (qualifications and experience)
	Neil Anderton	sheep	25 years of experimental use of sheep	restraining during vaccination and bleeding	practical experience in sheep handling, bleeding over 25 years

	Yu Cao	sheep	13 years	Vaccination and assistance in bleeding. Monitoring animals for well being.	Yu Cao took part in number of vaccination trials in the past. She is experienced in vaccine preparation and antibody titres assessment.
	Steve Colegate	sheep	26 years	administration of vaccine. Bleeding and well being monitoring of animals.	As a project leader and an expert in sheep trials Steve will oversee and supervise all procedures required to complete the experiment.
	Agnieszka Michalewicz	sheep	13 years	Vaccination and assistance in bleeding. Monitoring animals for well being.	Agnieszka took part in number of vaccination trials in the past. She is proficient in vaccine preparation and antibody titres assessment.
	Sandy Matheson	Sheep	Forty years	animal welfare, management, husbandry	Werribee LAF Manager
	Noel Collins	Sheep	25 years	animal welfare, management, husbandry, vaccination and bleeding	Werribee Farm, senior personnel

9.2	**Person responsible for day to day husbandry of animals (for example the facility manager):**
Sandy Matheson	
9.3	**Has this person been consulted on animal care for this project?**
Yes	

HUSBANDRY

10.1	**What is the feeding regime?**
grass, supplemented with other feed if necessary	
10.2	**What special housing requirements will be needed?**
none	
10.3	**What bedding or litter will be provided?**
Paddocked, no bedding needed	
10.4	**What environmental enrichment will be provided?**
Natural environment of the farm paddocks. No other enrichment will be provided.	
10.5	**How long will animals be held?**
2 years	

AEC1

PROJECT DESIGN

11.1	**Please outline the project design in terms of the different animal groups and for each group indicate its size (no. of animals) and the procedures to be performed:**

Study 1 -Determination of the Kinetics and Nature of Corynetoxin Antibody Titres- 120 sheep
12 groups of 10 sheep

Study 2 –Dose volume and Single Booster- 20 sheep
2 groups of 10 sheep

Study 3- Establishing Vaccine Stability after 6 and 12 month storage -60 sheep
6 groups of 10 sheep

The procedures in Study 1, 2 and 3 include vaccination and bleeding only. In the following table, "V" represents the time of vaccination (primary and subsequent boosters). The best performing protocol from Study 1 will be utilized in Study 2 and Study 3.

11.2	**Please provide a table showing the treatment groups and animals per group:**

ARGT Vaccine Investigation Timeline

Month of Investigation	**Study 1** (4 time-related groups of 10 sheep for each of 3 dose groups)	**Study 2**		**Study 3**	
		1 ml dose volume (1 group of 10 sheep at one dose group)	**No V3** (1 group of 10 sheep at one dose group)	**6 month Stability** (1 group of 10 sheep at each of 3 dose groups)	**12 month Stability** (1 group of 10 sheep at each of 3 dose groups)
0	1				
1	#V2				
2					
3	V3				
4		V1	V1		
5		V2	V2		
6				V1	
7		V3		V2	
8					
9				V3	
10					
11					
12				Finish	V1
13					V2
14					
15	2nd V1				V3
16					
17			2nd V1		
18	Finish*				Finish

19		2nd V1			
20					
21					
22		Finish	Finish		
23					
24					

*The actual Finish times will depend upon data obtained
merged cells indicate injection could occur within that time period

Total number of sheep required = 200

11.3	**Please outline the time sequence by which the procedures are to be performed on the animals:**

As shown in 11.2

STATISTICAL DESIGN AND ANALYSIS

12.1	**Give a brief description of the statistical design eg. Fully controlled experiment/semi-controlled/observational study/other:**

It has been estimated that perhaps 10% of sheep will not respond to the vaccine. Therefore, 10 sheep will be used in each group to allow for individual variation and ensure that non-responders do not compromise the group result.

12.2	**Have specific design features been incorporated to improve experimental precision without increasing animal usage eg. Repeated measurement, pairing:** If not, please explain why.

No. The animal usage per group is minimal and naive animals are required for each study.

12.3	**Will specific analysis features be used to improve experimental power without increasing animal usage e.g. Hidden replication?** If not, please explain why.

No. Not appropriate

12.4	**Should animals have to be removed from the study for welfare reasons, how will this effect interpretation of the study?**

There is sufficient number of animals in each group to allow for unforeseen, minor losses for treatment unrelated reasons.

12.5	**Please outline how the results of any intended statistical analysis will be used to assess whether the study objectives have been achieved:**

The main object is to demonstrate an increase in the corynetoxin antibody titres following vaccination. This increase should be obviously significant if it is to provide protection against the disease.

EXPERIMENTAL PROCEDURES

13.1 Proposed vaccination and/or Challenge Inoculation Schedule:

Species	sheep			
Agent	hapten-conjugate			
Route	subcutaneous			
No. of sites	neck side (alternated)			

	Volume	1 and 2 ml			
	Needle gauge	18 G			
	Frequency	As shown in 11.2			
	Adjuvant	Triple Adjuvant			
	Sedation	N/A			
	Anaesthetic	N/A			
	Restraint	manual			
	SOP Number(s) and Synopsis (1-2 sentences)				
13.2	**Bleeding**				
	Species	sheep			
	Body weight	30-60 kg (monthly monitoring)			
	Route	Jugular vein			
	No. of sites	Both sides			
	Volume	10 ml			
	Needle gauge	18G			
	Frequency	weekly			
	Sedation	N/A			
	Anaesthetic	N/A			
	Restraint	Manual			
	SOP Number(s) and Synopsis (1-2 sentences)				

AEC SOP #8 **Blood Sampling from Poultry, Sheep, Goats, Horses and Pigs**
AEC SOP #12 Blood Volume & Collection Guidelines
AEC #15 Environmental Enrichment

13.3 Are there other details relevant to the procedures to be carried out on animals that would assist the AEC in assessment of this protocol?

After each vaccination the animals will be examined by Werribee LAF staff for site reaction.

MONITORING AND INTERVENTION

14.1	**List the methods that will be used to monitor the animals:**		General wellbeing check-up by observation	Site reaction by palpation	
14.2	**How frequently will monitoring occur?**	**Non disease periods**	Daily		
		Diseases periods	N/A		

14.3 Describe the signs of disease expected in this project:

N/A

14.4	**Grade the signs to reflect the appearance of mild, moderate and severely affected animals:**	
	Mild	None to be expected
	Moderate	None to be expected
	Severe	None to be expected

14.5 What is the scientific end point of the experiment?

To answer the following questions:

- The optimum time for giving the booster injection of the ARGT vaccine after the first injection. *Timing the second injection for just after the peak of antibody titre following the primary injection is expected to result in the best boost to corynetoxin antibody levels.*
- The optimum concentration of immunogenic conjugate required to stimulate the highest and most stable (persistent) level of corynetoxin antibodies i.e., the kinetics of the antibody production
- Whether a secondary booster injection is required
- Which of two volumes of vaccine formulation produces the better antibody titre kinetics and the best memory effect after 12 months
- Which of 3 immunogenic conjugate levels provides the most stable vaccine when stored for 6 months and 12 months.
- Which antibody isotypes are produced? Does the IgG get re-induced after 12 months or is it a re-induction of IgM mainly?

14.6 With respect to disease severity at what point will you intervene/euthanase?
(Note that this should be consistent with achieving scientific end point)

No disease related to the vaccination is expected

14.7 Please attach a copy of the animal monitoring sheet:

14.8 What measures will be taken if animal wellbeing is compromised for unforeseen reasons?

euthanasia

14.9 If applicable, what pharmacological measures will be used to minimise pain or distress?

Include drug name, dose and method of administration of anaesthetic or tranquillising agents to be used. In addition, for analgesics please specify when they will be used and expected duration of analgesia.

Drug name	N/A			
Dose				
Route				
Expected Duration				
Frequency				
Other Comments				

EUTHANASIA

15.1 Method of euthanasia:

captive bolt

15.2	**If using pharmacological agents please provide the following details:**			
	Drug name	N/A		
	Dose (mg/kg)			
	Route			

15.3	**Describe measures to reduce pain or distress during this procedure:**

OTHER WELFARE ISSUES

16.1	Please outline any other issues impacting on welfare:

Ensure good animal handling practice and adhere to the SOP related to administration of the vaccine, bleeding and animal welfare

DECLARATION

17.1	**"I agree to carry out this experiment in accordance with the above protocol and the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes' 2004"**
17.2	**Application submitted by:**

Please forward a copy of completed form as an email Word attachment via Microsoft Outlook to Tim Hancock.

5.2 Standard Operating Procedures

5.2.1 Vaccine Preparation

5.2.1 Preparation of Immunogen for ARGT Vaccine

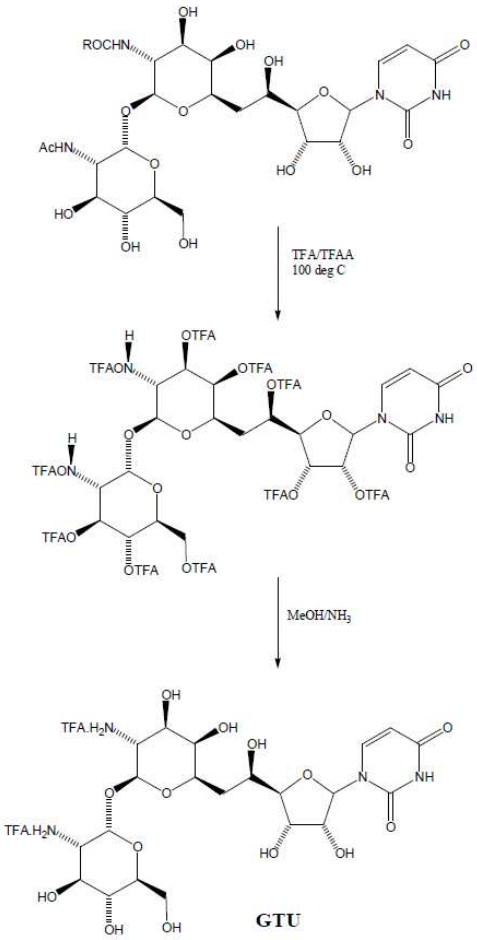
The immunogen used in the ARGT vaccine was prepared in a two part process.

1. Glucosaminyltunicaminyl Uracil (GTU) was prepared by cleaving the amide linkages in tunicamycin and trifluoroacetylating all hydroxyl and amine functionality. The trifluoroacetyl esters formed were hydrolysed using ammonia in methanol, and the GTU was purified as the trifluoroacetyl salt.
2. The purified GTU.2TFA was then reacted with Foetal calf serum to form the FCS-GTU conjugate used in the vaccine preparation

1. Preparation of Glucosaminyltunicaminyl Uracil (GTU)

Tunicamycin can be converted to GTU in a two step process. Firstly Tunicamycin was deca-trifluroacetylated by heating at 100 deg. C with a mixture of trifluroacetic anhydride and trifluroacetic acid. In view of the reagents volatility, the reaction was undertaken in a pressure vessel and allowed to react for a period of 2 days. The excess reagent was removed and the residue washed with pentane to remove the lipophilic components.

Scheme 1



GTU

Saturated Ammonia in Methanol was then added and allowed to react at room temperature for 7 days. The excess solvent and reagent were removed under reduced pressure and the residue purified on a preparative C8 HPLC column using 0.1% TFA in water with 260 nm detection (PDA detector).

Experimental

Synthesis of Glucosaminyltunicaminyl Uracil ditrifluoroacetate (GTU.2TFA)

Tunicamycin (TM) (up to 200 mg) Trifluroacetic anhydride (30 mL) and Trifluroacetic acid (0.60 mL) were sequentially added to a 50 mL Teflon lined Parr hydrogenation Bomb reactor and sealed with the screw cap to the manufacturers specifications. The reactor was heated at 100C for 48 hrs .
The reactor was allowed to cool to room temperature. The contents were transferred to a 250 ml RB flask with a stirrer bar and a rapid stream of nitrogen blown into the stirred solution in a fume hood, until no liquid remained (approx. 20 mins). The resultant oily residue was treated with pentane (2x50 mL) to remove the lipophilic functionality cleaved from TM in the first step of this process. (sonication helps in the dissolution of the fatty acids at this stage). The residue was dried under vacuum generating a pale brown solid that was then directly treated with saturated ammonia in methanol (100 mL, approx 18 M) with stirring for a period of 7 days. The reaction during this stage was protected from light by wrapping in foil.

After 7 days the NH3 was removed blowing nitrogen over the surface of the solution (checked by using a wetted pH paper on the vapour "blow off") Removal of the ammonia took approx. 2 hours. At this stage the solution was acidified to pH 3-4 using trifluroacetic acid (TFA) then solvent removed under reduced pressure. After removal of the methanol, the flask was placed under vacuum pump pressure to remove any residual TFA leaving a brown, glassy film should in the flask (at this stage the GTU was in the form GTU.2TFA). The residue was dissolved in the minimum amount of 0.1% TFA in water (5 mL).

HPLC: The GTU fraction, in 5 ml 0.1% TFA, was sonicated for 2 x 2 min then filtered through a 0.45 um 13 mm syringe filter. The flask was rinsed with 1x 2.5 ml 0.1% TFA and the rinsings filtered through the 0.45 um filter. The sample was applied to the column using a 2.5 ml glass Luer Lock syringe and a Luer lock adapter fitted to the column. The column was connected to Shimadzu HPLC and a fraction collection run performed. The GTU fraction (approx tubes12-18) were combined, evaporated to dryness, redissolved in MeOH, dried then suspended in EtOAc. The EtOAc was removed in vacuo and the solid residue dried under vacuum. A creamy-white powder was obtained on drying. The suspension in ethyl acetate step is required as the product after evaporation of methanol is harder to remove from the flask and more gum-like, whereas the product after the ethyl acetate is easily removed and forms a free flowing powder.

The powder from multiple runs was combined, dissolved in methanol, dried in vacuo, resuspended in ethyl acetate then dried in vacuo to homogenise the product. The GTU.2TFA was dried under vacuum to remove traces of ethyl acetate.

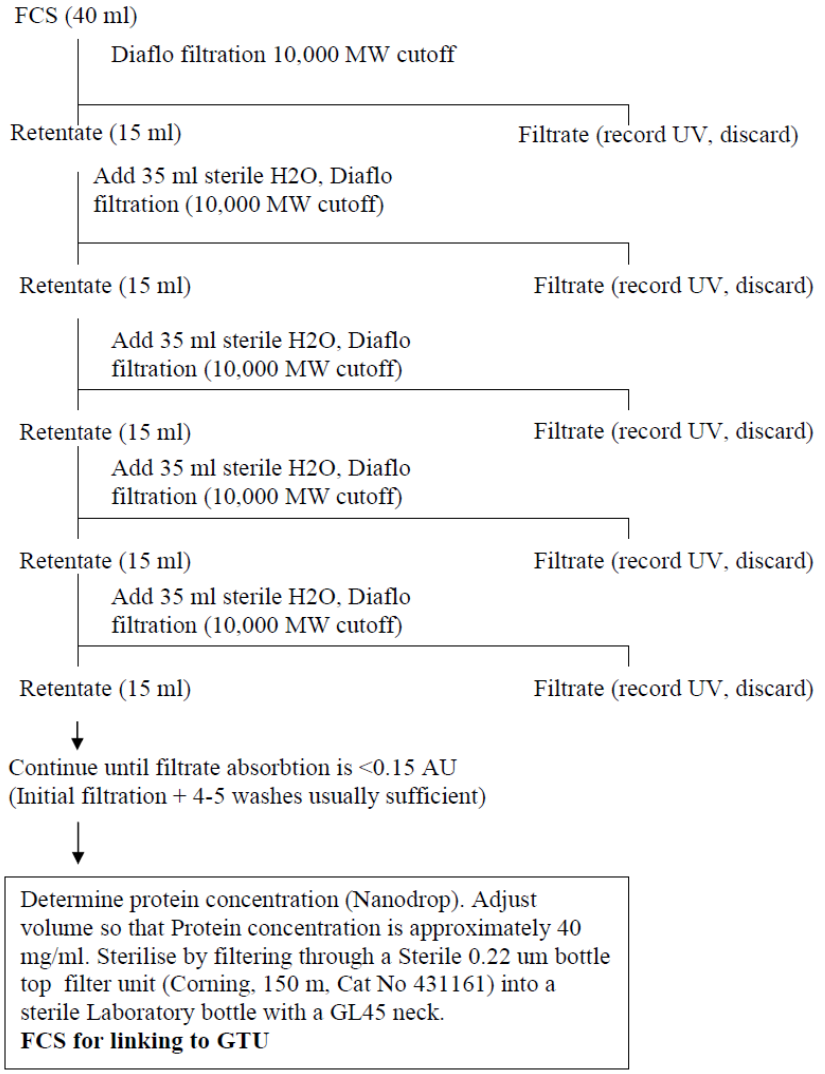
2. Preparation of Immunogen

Removal of Low Molecular Weight (<10,000) UV absorbing components from Foetal Calf Serum

Source: Foetal Calf Serum (FCS, AAHL Cell culture stock – Australian origin). Determine the protein concentration using the UV protein analysis procedure (Nanodrop Spectrometer). The AAHL stock FCS should be between 25 and 35 mg protein/ml

Cleanup: The FCS (40 ml) was filtered through a 10000 MW cutoff Diaflo filter to remove UV absorbent compounds from the protein solution. The initial filtrate was processed until the volume of retentate was reduced to 15 ml. 35 ml of sterile water was added, and the process repeated until the UV absorbance of the filtrate was less than 0.15 AU.

The FCS was treated as follows:



Note: sterile water was not used to preserve sterility, but to minimise microbiological contaminants.

Conjugation of GTU to FCS
Reagents: Quantity per 100 mg protein
(Reagent quantities can be scaled up or down as required)

GTU concentration used is 5 X GTU expected to bind to the protein (empirically determined):
GTU: 2 mg/ml solution in water. Prepare from 85% pure GTU.2TFA
*To calculate quantity of GTU.2TFA required: Mg GTU.2TFA = (Mg GTU)/0.85*794/566*
*Protein concentration determined using NanoDrop Spectrometer refer to "*Direct determination of GTU linked to protein" *section below.*
794 and 566 are the molecular weights for GTU.2TFA and GTU respectively

Reagents:
GTU: 8 mg (ex 2 mg/ml solution)
FCS: 100 mg (2.5 ml if protein concentration 40 mg/ml)
EDC: 125 mg (12.5 ml at 10 mg/ml conc)
Water As required to make to total volume of 23 ml

The GTU, FCS and EDC solutions were mixed in an appropriately sized laboratory bottle. A sterile bottle top filter was screwed onto a sterile GL45 neck laboratory bottle under sterile conditions (Laminar Flow Cabinet), and the reaction mixture filtered into the bottle (once the filter is attached, filtration step may be performed on the open bench or Fumehood. The bottle top filter was removed under sterile conditions and a sterilized stirrer bar added. The bottle cap was attached, the reaction bottle covered in foil to exclude light and the reaction mixture stirred for 44 hours at room temperature on a magnetic stirrer.

The reaction bottle was removed from the magnetic stirrer and the contents transferred to an appropriately sized Diaflo Filtration unit with 10000 MW cutoff filter. Pressure was applied (60 psi max) and the filtrate collected. Filtration was stopped when a small quantity of solution remained above the filter (~10 ml for the 50 ml unit, ~25 ml for the 250 ml unit). The filtrates were retained and the UV spectrum recorded.
Water was added to the Diaflo unit (35 ml for the 50 ml unit, 150 ml for the 250 ml unit) and the filtration repeated until the UV absorbtion of the Diaflo filtrate was <0.15 AU (usually 4-5 washes).
The retentate was removed from the Diaflo filtration unit (filter unit washed 3 x with water - ~5 ml/wash for the 50 ml unit, ~15 ml/wash for the 250 ml unit), the retentate and washings combined, the protein concentration measured (NanoDrop) and adjusted to ~4mg/ml (concentration required for vaccine formulation).
After the protein concentration was adjusted to ~4 mg/ml, the solution was sterile filtered through a Sterile 0.22 um bottle top filter unit (Corning, 150 m, Cat No 431161) into a sterile Laboratory bottle with a GL45 neck.
This solution was kept (1-2 weeks) at 4°C until used for initial vaccine formulation. The remainder of the sterilized FCS-GTU conjugate was be dispensed into appropriate aliquots (under sterile conditions) for future formulations of the vaccine and the sealed tubes stored at -20°C until use.

Direct determination of GTU linked to protein:
The Protein determination method used by the NanoDrop Spectrometer also reports the absorbance ratio A260/A280, which is used for DNA determination. This can also be used to calculate the amount of GTU linked to the protein, as GTU also absorbs at 260 nm.
Assumptions:

- The GTU absorbtion remains at 260 nm, with an ϵ_{260} of 9650. Prior experience with Glucosaminyltunicaminyluracil antibiotics and their derivatives supports this assumption.
- The GTU does not contribute significantly to absorbtion at 280 nm (actually contribution is ~10% of the 260 absorbtion of GTU). The GTU attachment will be slightly underestimated due to this contribution.

To determine the attachment of GTU to the protein, the protein was diluted 20 fold (to about the same protein concentration as the diaflo retentates of the GTU-FCS product), assayed using the Nanodrop UV spectrometer, and the 260/280 ratio recorded (~0.61)
The protein concentration and ratio was then recorded for the GTU-FCS preparations.
The difference in UV absorbtion at 260 nm (A260 GTU-FCS – A260 FCS) was calculated, assuming an ϵ_{260} = 9650 and a molecular weight of 566, and used to determine the amount of GTU linked to the protein (refer Excel spreadsheet O:\MOL\ptu\ARGTvaccine\GTU\ARGT Vaccine Trial 2007 GTU-FCS conjugation.xls).

5.2.2 Corynetoxin Antibody ELISA

5.2.2 Anti-corynetoxin antibody titre assay for sheep serum

Procedural notes

1. Prepare all the reagents and samples before beginning the assay.
2. When preparing the buffered solutions, pH meter must be use to check the pH of each solution. Final pH of each solution must be within ± 0.2 of the specified pH.
3. All reagents should be allowed to warm to room temperature (20-25°C) before use.
4. Prepare only the volume of each diluted reagent necessary for the batch of samples under test. Do not keep unused diluted reagents longer than the specified period.
5. The wash procedure is critical. Insufficient washing may affect the intensity of colour development in the wells.
6. Precise pipetting of reagents is essential. A new tip must be used for each reagent or sample.

Equipment, Material, Reagents and Storage

Plate washer or Nunc Immuno wash 12 (manual wash)
Nunc-Immuno plate, F96 Cert. MaxiSorp 439454

1. Sodium bicarbonate ($NaHCO_3$)
2. Sodium carbonate (Na_2CO_3)
3. Glutaraldehyde ~25% solution use for plate treatment kept at 4°C

4. Glucosaminyltunicaminyluracil ditrifluoroacetate (GTU.2TFA) for coating (Coating solution 10 µg/ml in 50% methanol or 10 part per million) at -18°C

5. Sodium chloride (NaCl)

6. Bovine serum albumin (BSA) for assay buffer at 4°C
7. Phosphate buffered saline tablets (PBS, Oxoid Code BR14 a)
8. Tween 20 (Polyoxyethylenesorbitan Monolaurate, Sigma P1379)

9. Anti-corynetoxin antisera for testing in 1/10 dilution (50 µl antisera and 0.45 ml of PBS /0.05% thimerosal) at 4°C.
10. Donkey anti-sheep IgG-horseradish peroxidase enzyme conjugate (DAS-HRP 1/10) in 50% glycerol buffer/PBS with 0.1% thimerosal at 4°C.
11. K-Blue TMB Substrate (Graphic Scientific Pty Ltd.) at 4°C.

12. 0.5 M sulphuric acid (H_2SO_4)

13. thimerosal, Sigma, Lot 127H1481

14. Glycerol

Buffer for glutaraldehyde treatment (0.1 M Carbonate buffer, pH 9.0)

1. Dissolve 0.84 gm of sodium bicarbonate in 100 ml reagent grade water (pH ~8.4).
2. Dissolve 0.53 gm of sodium carbonate in 50 ml reagent grade water (pH~11.1).
3. Adjust pH of 1 (sodium bicarbonate) to 9.0 by adding 2 (sodium carbonate). (will need about 10.6 ml sodium carbonate for 100 ml sodium bicarbonate. Check the pH by using pH meter). Final pH of the solution must be within ± 0.2 of the specified pH (9.0).
4. Store at 4°C and use within 4 weeks.

Washing solution (10x)

1. Dissolve 438 gm of sodium chloride (NaCl) and 25 ml of Tween 20 in 5 L reagent grade water.
2. Stored at 4°C use within 6 months.

Washing solution (1x)

1. Mix 500 ml of 10x washing solution with 4.5 L of l reagent grade water.
2. Stored at 4°C, use within 2 weeks.

Sera storage buffer (PBS/0.05% thimerosal)

1. Dissolve one tablet of Oxoid phosphate buffered saline and 0.05 gm of thimerosal in 100 ml of reagent grade water.
2. Stored at 4°C use within 6 months
3. Pipette each 50 µl of sera into 0.45 ml sera storage buffer (1/10) stored at 4°C for use.

Assay buffer (0.5% BSA and 0.05% Tween 20 in PBS, pH 7.3)

1. Dissolve one tablet of Oxoid phosphate buffered saline in 100 ml of reagent grade water.
2. Dissolve 50 µl of Tween 20 in 100 ml of phosphate buffered saline.
3. Dissolve 0.5 gm of BSA in 100 ml of 0.05% Tween 20 in phosphate buffered saline.
4. Store at 4°C, use within 2 weeks.

DAS-HRP storage buffer (50% Glycerol/PBS/0.01 % thimerosal)

1. Dissolve one tablet of Oxoid phosphate buffered saline in 50 ml of reagent grade water and 50 ml of glycerol.
2. Dissolve 0.01 gm of thimerosal in 100 ml of 50 % glycerol/ phosphate buffered saline.

Stopping solution (0.5 M H_2SO_4)

(1:36 dilution of conc. H_2SO_4)

1. Add 25 ml of Conc. H_2SO_4 into 875 ml of pure water.
2. Store at room temperature, use within 6 months.

Treatment of ELISA plates

The plates are first treated with 0.2% glutaraldehyde before coating with (GTU.2TFA). The glutaraldehyde treatment prepares the surface of the plates for coating with small molecular weight compounds, such as Glucosaminyltunicaminyluracil ditrifluoroacetate that do not readily coat on non-treated surfaces of ELISA plates. Prepare 0.2% glutaraldehyde fresh; do not store the diluted glutaraldehyde.

For 10 ELISA plates:

1. Dissolve 0.8 ml of 25% glutaraldehyde (stock commercial solution) in 99.2 ml carbonate buffer, pH 9.0.
2. Add 0.1 ml of 0.2% glutaraldehyde in pH 9 carbonate buffer/well of ELISA plate.
3. Cover the plates, place the plates as one-layer on the rack in the incubating oven and incubate for 2 hours at 56°C. Do not stack the plates during the incubation.
4. Wash the plate 4x with pure water to remove excess glutaraldehyde.

Coating of ELISA plates with chemically modified tunicamycin derivative (GTU.2TFA).

1. Prepare 1/250 dilution of chemically modified tunicamycin (stock is 10 µg/ml in 50% methanol) in reagent grade water.
   [ie. For 10 plates - add 400 µl of 10 µg modified tunicamycin/ml to 99.6 ml of water]
2. Pipette 100 µl of diluted 40 ng/ml modified tunicamycin into each well (4 ng/0.1 ml/well) of flat-bottom 96-well microtitre plates (coat only row 1 to 12).
3. Cover and incubate the plates at 56°C for 2 hours followed by 4 °C.
4. After 40 to 48 hours coating at 4°C, the plates can be used the same day or cover the plate with plate cover or sealer and store at 4°C for 1-2 weeks.

Protocol for serum titration

The plate must already be treated with 0.2% glutaraldehyde and coated with 4 ng/well of GTU.2TFA. All steps are conducted at room temperature (20-25°C).

1. Wash the 4 ng/well of GTU.2TFA coated plates 4x with 300 µl/well of wash solution. During the washing procedure, turn the plate upside down and shake out the contents of the wells by striking the plate firmly on paper towel.
2. Map 10 assay sera in 12X8 ELISA plate format with control sera negative and positive position at row 1 and 2.
3. Warm the assay sera in 1/10 storage solution at 4 °C to room temperature.
4. Add 100 µl assay buffer (0.5% BSA and 0.05% Tween 20 in PBS, pH 7.3) to each well for whole plate and Column A from row 1 to 12 add additional 80 µl (in volume 180 µl).
5. Pipette each 20 µl of assay sera (1/10 in storage solution) into the wells (with 180µl assay buffer) from column A row 1 to 12 (follow the ELISA plate format).

6. Use12 channels pipette. Fill up all the plates you are going to use for the day before you start adding the diluted sera into the plate.

7. Use12 channels pipette sucking in and out (aspirating/dispensing) for 6 times at Column A, pipette 100 µl of diluted sera to the wells of row 2 and mix so on till column H.

8. Cover the plate (use separate cover for each plate), and incubate at room temperature for 2 hours.

9. After incubation, wash the plate 4 times with washing solution as for step 1.

10. Pipette 100 µl of 1/5000 or 1/8000 dilution of anti- sheep IgG-horseradish peroxidase in assay buffer into each well of row 1 to 12.
    [ie. For 1 plate: in 1/8000 dilution add 12.5µl of stock anti-sheep IgG-horseradish peroxidase (1/10) to 9.987 ml of 0.5% BSA and 0.05% Tween 20 in PBS assay buffer. Do not store the diluted anti-sheep IgG-horseradish peroxidase enzyme conjugate.]

11. After 1 hour incubation, wash the plate 4 times with washing solution as for step 1.

12. K-Blue TMB substrate solution:
    Immediately prior to use, prepare K-Blue TMB substrate solution in a suitable container. Warm up 15 ml of K-Blue TMB substrate.

13. Add 100 µl of K-Blue TMB substrate solution into each well of row 1 to 12.

14. After incubation of 20-30 minutes, stop the colour reaction by adding 50 µl/well of 0.5 M sulphuric acid.

15. Shake for 1 minute on a microtitre plate shaker or gently hold the side of the plate and shake by hand manually.

16. Measure the optical density at 450 nm.

17. Plot the optical density (Y axis) against the dilution of the serum (X axis) or use the computer program to calculate anti-corynetoxin antibody titre of the samples.

Checking the binding of the antiserum with tunicamycin

If the antiserum, after diluted 1/1000 or more, gave optical density higher than 1.0 in titration assay, antiserum should be checked for the binding with free tunicamycin.

Prepare 1 ng/0.1 ml tunicamycin standard by 1/100 dilution of the tunicamycin 1 µg/ml in assay buffer (ie. For 1 plate: 70 µl of 1 µg/ml stock add to 6.93 ml assay buffer)

The plate must already be treated with 0.2% glutaraldehyde and coated with 4 ng/well of chemically modified tunicamycin (row 2-11). All steps are conducted at room temperature (20-25°C).

1. Thaw, vortex and pipette 50 µl of antisera for testing into 10 ml assay tubes.

2. Add 4.95 ml of 0.05% thimerosal (preservative) in PBS, pH 7.3 (1/100 dilution) and vortex.

3. Add 200 µl of the 1/100 serum in step 2 to 1.8 ml assay buffer. This gives a solution of 1/1000 dilution.

4. Prepare four further two-fold serial dilutions by mixing 1 ml of diluted serum with 1 ml of assay buffer to get the following dilutions:
   1/2000 dilution
   1/4000 dilution
   1/8000 dilution
   1/16,000 dilution

5. Wash the 4 ng/well of chemically modified tunicamycin (GTU) coated plates 4x with 300 µl/well of wash solution. During the washing procedure, turn the plate upside down and shake out the contents of the wells by striking the plate firmly on paper towel.

6. Pipette 100 µl of assay buffer into column A, B, E and F of row 2 to 11.

7. Pipette 100 µl of 1 ng/0.1 ml tunicamycin standard into column C, D, G and H of row 2 to 11.

8. Add 50 µl diluted serum into the wells of row 2 to 11 (see the example of the ELISA plate format).

9. Shake for 1 minute on a microtitre plate shaker or gently hold the side of the plate and shake by hand manually.

10. Cover the plate (use separate cover for each plate), and incubate at room temperature for 2 hours.

11. After incubation, wash the plate 4x with washing solution as for step 3.

12. Pipette 100 µl of 1/5000 dilution of anti- sheep IgG-horseradish peroxidase in assay buffer into each well of row 2 to 11.

[ie. For 1 plate: add 80 µl of stock anti-sheep IgG-horseradish peroxidase (1/40) to 9.92 ml of 0.5% BSA and 0.05% Tween 20 in PBS assay buffer. Do not store the diluted anti-sheep IgG-horseradish peroxidase enzyme conjugate.]

13. After 1 hour incubation, wash the plate 4x with washing solution as for step 3.

14. For in-house TMB substrate solution:
    Immediately prior to use, prepare TMB substrate solution in a suitable container. Warm up 15 ml of substrate buffer pH 5.5 to 30°C. Add 225µl TMB 10 mg/ml of DMSO solution (1.5%) and mix, then add 2.25 µl of 30% hydrogen peroxide (H2O2) (0.015%).

15. Add 100 µl of TMB in-house substrate solution or "K-Blue TMB Substrate", from Graphic Scientific Pty Ltd, into each well of row 1 to 11.

16. After incubation of 20-30 minutes, stop the colour reaction by adding 50 µl/well of 0.5 M sulphuric acid.

17. Shake for 1 minute on a microtitre plate shaker or gently hold the side of the plate and shake by hand manually.

18. Measure the optical density at 450 nm.

19. Plot the optical density (Y axis) against the amount of dilution of the serum (X axis) and compare of the curves with 1 ng/0.1 ml tunicamycin standard and without tunicamycin.

Chemical requirements

15. Glutaraldehyde ~25% solution
16. Phosphate buffered saline tablets (PBS, Oxoid Code BR14 a)
17. Sodium bicarbonate ($NaHCO_3$)
18. Sodium carbonate (Na_2CO_3)
19. Sodium chloride (NaCl)
20. Sulphuric acid (H_2SO_4)
21. Thimerosal (Sigma T5125)
22. Tween 20 (Polyoxyethylenesorbitan Monolaurate, Sigma P1379)
23. For in-house TMB substrate (optional)
    - Citric acid monohydrate, (COOH. CH_2. C(OH).COOH. CH_2. COOH. H_2O)
    - Dimethylsulfoxide, DMSO
    - 30 % hydrogen peroxide, H_2O_2
    - Sodium acetate trihydrate (CH_3.COONa $3H_2O$)
    - TMB (Tetramethylbenzidine, Sigma T-2885)

(Alternative TMB substrate is "K-Blue TMB Substrate" Cat: 300177 from Graphic Scientific Pty Ltd.)

Other requirements

1. Flat-bottom 96-well microtitre plates (Nunc 439454, Cert.Maxisorp)
2. Pipettes:
   1-5 ml pipette for dilution of standards and samples
   100 µl pipette
   50-100 µl eight channels pipette
   1-20 µl pipette for in-house substrate solution
3. Plate cover
4. Plate sealers (Acetate, ICN Biochemicals Inc. 76-401-05) (optional)
5. ELISA plate shaker (optional)
6. ELISA plate reader
7. ELISA plate washer (manual or automatic)
8. Reagent basin
9. 10 ml tubes for dilution of corynetoxin standard and samples

5.2.3 Antibody Isotyping ELISA

Anti-corynetoxin antibody isotyping assay for sheep serum

Procedural notes

1. Prepare all the reagents and samples before beginning the assay.
2. When preparing the buffered solutions, pH meter must be use to check the pH of each solution. Final pH of each solution must be within ± 0.2 of the specified pH.
3. All reagents should be allowed to warm to room temperature (20-25°C) before use.
4. Prepare only the volume of each diluted reagent necessary for the batch of samples under test. Do not keep unused diluted reagents longer than the specified period.
5. The wash procedure is critical. Insufficient washing may affect the intensity of colour development in the wells.
6. Precise pipetting of reagents is essential. A new tip must be used for each reagent or sample.

Equipment, Material, Reagents and Storage

Plate washer or Nunc Immuno wash 12 (manual wash)
Nunc-Immuno plate, F96 Cert. MaxiSorp 439454

1. Sodium bicarbonate ($NaHCO_3$)
2. Sodium carbonate (Na_2CO_3)
3. Glutaraldehyde ~25% solution use for plate treatment kept at 4°C

4. Glucosaminyltunicaminyluracil ditrifluoroacetate (GTU.2TFA) for coating (Coating solution 10 µg/ml in 50% methanol or 10 part per million) at -18°C

5. Sodium chloride (NaCl)

6. Bovine serum albumin (BSA) for assay buffer at 4°C
7. Phosphate buffered saline tablets (PBS, Oxoid Code BR14 a)
8. Tween 20 (Polyoxyethylenesorbitan Monolaurate, Sigma P1379)

9. Anti-corynetoxin antisera for testing in 1/10 dilution (50 µl antisera and 0.45 ml of PBS /0.05% thimerosal) at 4°C.
10. IgG1 1.39 mg/ml
11. IgG2 1.59 mg/ml
12. IgM 0.9 mg/ml
13. Ig-HRP
14. K-Blue TMB Substrate (Graphic Scientific Pty Ltd.) at 4°C.

15. 0.5 M sulphuric acid (H_2SO_4)

16. thimerosal, Sigma, Lot 127H1481

17. Glycerol

Buffer for glutaraldehyde treatment (0.1 M Carbonate buffer, pH 9.0)

1. Dissolve 0.84 gm of sodium bicarbonate in 100 ml reagent grade water (pH ~8.4).
2. Dissolve 0.53 gm of sodium carbonate in 50 ml reagent grade water (pH~11.1).
3. Adjust pH of 1 (sodium bicarbonate) to 9.0 by adding 2 (sodium carbonate). (will need about 10.6 ml sodium carbonate for 100 ml sodium bicarbonate. Check the pH by using pH meter). Final pH of the solution must be within ± 0.2 of the specified pH (9.0).
4. Store at 4°C and use within 4 weeks.

Washing solution (10x)

1. Dissolve 438 gm of sodium chloride (NaCl) and 25 ml of Tween 20 in 5 L reagent grade water.
2. Stored at 4°C use within 6 months.

Washing solution (1x)

1. Mix 500 ml of 10x washing solution with 4.5 L of l reagent grade water.
2. Stored at 4°C, use within 2 weeks.

Sera storage buffer (PBS/0.05% thimerosal)

1. Dissolve one tablet of Oxoid phosphate buffered saline and 0.05 gm of thimerosal in 100 ml of reagent grade water.
2. Stored at 4°C use within 6 months
3. Pipette each 50 µl of sera into 0.45 ml sera storage buffer (1/10) stored at 4°C for use.

Assay buffer (0.5% BSA and 0.05% Tween 20 in PBS, pH 7.3)

1. Dissolve one tablet of Oxoid phosphate buffered saline in 100 ml of reagent grade water.
2. Dissolve 50 µl of Tween 20 in 100 ml of phosphate buffered saline.
3. Dissolve 0.5 gm of BSA in 100 ml of 0.05% Tween 20 in phosphate buffered saline.
4. Store at 4°C, use within 2 weeks.

DAS-HRP storage buffer (50% Glycerol/PBS/0.01 % thimerosal)

1. Dissolve one tablet of Oxoid phosphate buffered saline in 50 ml of reagent grade water and 50 ml of glycerol.
2. Dissolve 0.01 gm of thimerosal in 100 ml of 50 % glycerol/ phosphate buffered saline.

Stopping solution (0.5 M H_2SO_4)

(1:36 dilution of conc. H_2SO_4)

1. Add 25 ml of Conc. H_2SO_4 into 875 ml of pure water.
2. Store at room temperature, use within 6 months.

Treatment of ELISA plates

The plates are first treated with 0.2% glutaraldehyde before coating with (GTU.2TFA). The glutaraldehyde treatment prepares the surface of the plates for coating with small molecular weight compounds, such as Glucosaminyltunicaminyluracil ditrifluoroacetate that do not readily coat on non-treated surfaces of ELISA plates. Prepare 0.2% glutaraldehyde fresh; do not store the diluted glutaraldehyde.

For 10 ELISA plates:

1. Dissolve 0.8 ml of 25% glutaraldehyde (stock commercial solution) in 99.2 ml carbonate buffer, pH 9.0.
2. Add 0.1 ml of 0.2% glutaraldehyde in pH 9 carbonate buffer/well of ELISA plate.
3. Cover the plates, place the plates as one-layer on the rack in the incubating oven and incubate for 2 hours at 56°C. Do not stack the plates during the incubation.
4. Wash the plate 4x with pure water to remove excess glutaraldehyde.

Coating of ELISA plates with chemically modified tunicamycin derivative (GTU.2TFA).

1. Prepare 1/250 dilution of chemically modified tunicamycin (stock is 10 µg/ml in 50% methanol) in reagent grade water.
   [ie. For 10 plates - add 400 µl of 10 µg modified tunicamycin/ml to 99.6 ml of water]
2. Pipette 100 µl of diluted 40 ng/ml modified tunicamycin into each well (4 ng/0.1 ml/well) of flat-bottom 96-well microtitre plates (coat only row 1 to 12).
3. Cover and incubate the plates at 56°C for 2 hours followed by 4 °C.
4. After 40 to 48 hours coating at 4°C, the plates can be used the same day or cover the plate with plate cover or sealer and store at 4°C for 1-2 weeks.
5. (IgM plate no GTU)

Protocol for serum titration

The plate must already be treated with 0.2% glutaraldehyde and coated with 4 ng/well of GTU.2TFA. All steps are conducted at room temperature (20-25°C).

1. Wash the 4 ng/well of GTU.2TFA coated plates 4x with 300 µl/well of wash solution. During the washing procedure, turn the plate upside down and shake out the contents of the wells by striking the plate firmly on paper towel.
2. Map 12 assay sera in 12X8 ELISA plate format.
3. Warm the assay sera in 1/10 storage solution at 4 °C to room temperature.
4. Add 100 µl assay buffer (0.5% BSA and 0.05% Tween 20 in PBS, pH 7.3) to each well for whole plate and Column A from row 1 to 12 add additional 80 µl (in volume 180 µl).
5. Pipette each 20 µl of assay sera (1/10 in storage solution) into the wells (with 180µl assay buffer) from column A row 1 to 12 (follow the ELISA plate format).

6. Use12 channels pipette. Fill up all the plates you are going to use for the day before you start adding the diluted sera into the plate.

7. Use12 channels pipette sucking in and out (aspirating/dispensing) for 6 times at Column A, pipette 100 µl of diluted sera to the wells of row 2 and mix so on till column H.

8. Cover the plate (use separate cover for each plate), and incubate at room temperature for 2 hours.

9. After incubation, wash the plate 4 times with washing solution as for step 1.

10. Pipette 100 µl of 1/500 IgG1 or 1/1000 of IgG2 or 1/2000 of IgM in assay buffer into each well of row 1 to 12.

11. After 1 hour incubation, wash the plate 4 times with washing solution as for step 1.

12. Pipette 100 µl of 1/5000 Ig-HRP in assay buffer into each well of row 1 to 12.

13. After 1 hour incubation, wash the plate 4 times with washing solution as for step.

14. K-Blue TMB substrate solution:
    Immediately prior to use, prepare K-Blue TMB substrate solution in a suitable container. Warm up 15 ml of K-Blue TMB substrate.

13. Add 100 µl of K-Blue TMB substrate solution into each well of row 1 to 12.

14. After incubation of 20-30 minutes, stop the colour reaction by adding 50 µl/well of 0.5 M sulphuric acid.

15. Shake for 1 minute on a microtitre plate shaker or gently hold the side of the plate and shake by hand manually.

16. Measure the optical density at 450 nm.

17. Plot the optical density (Y axis) against the dilution of the serum (X axis) or use the computer program to calculate anti-corynetoxin antibody titre of the samples.

Chemical requirements

18. Glutaraldehyde ~25% solution
19. Phosphate buffered saline tablets (PBS, Oxoid Code BR14 a)
20. Sodium bicarbonate ($NaHCO_3$)
21. Sodium carbonate (Na_2CO_3)
22. Sodium chloride (NaCl)
23. Sulphuric acid (H_2SO_4)
24. Thimerosal (Sigma T5125)
25. Tween 20 (Polyoxyethylenesorbitan Monolaurate, Sigma P1379)
26. For in-house TMB substrate (optional)
    - Citric acid monohydrate, ($COOH.CH_2.C(OH).COOH.CH_2.COOH.H_2O$)
    - Dimethylsulfoxide, DMSO
    - 30 % hydrogen peroxide, H_2O_2
    - Sodium acetate trihydrate ($CH_3.COONa\ 3H_2O$)
    - TMB (Tetramethylbenzidine, Sigma T-2885)

(Alternative TMB substrate is "K-Blue TMB Substrate" Cat: 300177 from Graphic Scientific Pty Ltd.)

Other requirements

1. Flat-bottom 96-well microtitre plates (Nunc 439454, Cert.Maxisorp)
2. Pipettes:
   1-5 ml pipette for dilution of standards and samples
   100 µl pipette
   50-100 µl eight channels pipette
   1-20 µl pipette for in-house substrate solution
3. Plate cover
4. Plate sealers (Acetate, ICN Biochemicals Inc. 76-401-05) (optional)
5. ELISA plate shaker (optional)
6. ELISA plate reader
7. ELISA plate washer (manual or automatic)
8. Reagent basin
9. 10 ml tubes for dilution of corynetoxin standard and samples

5.3 Necropsy reports

Veterinary post mortem report

Plant Toxins ARGT vaccine trial, sheep # 123

Date: 31/3/2008

History: The Werribee staff have noticed this sheep decreasing in condition over the last few weeks. They have separated the sheep from the mob and have given it extra feed. The sheep continued to decrease condition which led Sandy Matheson to request it be euthanased and a necropsy completed.

Post mortem examination: The sheep was in thin body condition. No external lesions were noted. An abscess was detected on the medial side of the body of the left mandible, tracking up the ramus. There were no other gross abnormalities detected on gross necropsy.

Diagnoses/Implications: It is likely that this abscess is the cause of the loss of body condition in this sheep. This type of infection can be started from something like a grass seed penetrating this inside surface of the mouth.

If you have any queries, please let me know.

Regards,

Mark

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Veterinary post mortem report

Plant Toxins ARGT vaccine trial, sheep # 172

Date: 5/2/2008

History: This sheep was noticed lethargic on the evening of 4/2/2008. No previous observations of illness were observed. The sheep as a mob were observed earlier that day. This sheep was found dead first thing on 5/2/2008.

Post mortem examination: The sheep was in good body condition. No external lesions were noted. There were no gross abnormalities detected on post mortem.

Diagnoses/Implications: With this history and lack of post mortem findings commonly this is caused by one of the Clostridial diseases, such as Blackleg. These sheep have been vaccinated with a Clostridial vaccine, however some sheep will occasionally not respond appropriately to the vaccine.

If you have any queries, please let me know.

Regards,

Mark

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Veterinary post mortem report

Plant Toxins ARGT vaccine trial, sheep # 195

Date: 7/9/2008

History:

This sheep had been noticed in lighter than normal condition 10 days prior to the date of euthanasia. The sheep was separated into a smaller mob of sheep closer to the house and given extra feed, along with increased monitoring (at least twice daily). On examination a week after it was noticed losing condition there were no clinical signs evident other than the weight loss. At this time an injection of long acting antibiotics was given in case of an undiagnosed infection. When no improvement was seen 3 days after this injection the sheep was euthanased. At the time of euthanasia the sheep was in thin body condition but still bright and alert.

Post mortem examination:

At post mortem an abscess was detected at the back of the jaw

Diagnoses/Implications:

The injection of antibiotics was unable to sufficiently combat the infection. This can happen for a number of reasons, such as a tricky site of infection that antibiotics have trouble getting to, or the presence of a persistent foreign body (like a grass seed). The position of the abscess on the inside of the back of the jaw meant that no swelling was evident externally or on examination inside the mouth, making diagnosis difficult.

If you have any queries, please let me know.

Regards,

Mark

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