Final report

ANIMAL HEALTH AND WELFARE

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The development of a new or improved vaccine against *Boophilus microplus*: opportunities for R&D investment

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Abstract

The cattle tick causes severe economic losses to producers in northern Australia. Reliance on tick resistant cattle and chemical acaricides to solve the problem is increasingly problematic. Few, if any, alternative technologies are available. The original tick vaccine is no longer manufactured in Australia. An improved vaccine would provide an additional, desirable option for cattle producers and should be commercially attractive to a vaccine manufacturer.

This report describes the vaccine development pipeline, reviews the current, global status of research into tick vaccine development and describes the likely characteristics of a commercially attractive vaccine. Points at which investment by the MLA could facilitate the development of a second generation vaccine are identified. Currently these are in a more diversified approach to antigen identification and in examination of the potential of dual-antigen formulations. Should there be commercial interest in the reintroduction of a TickGARD-like vaccine in the short term; modes of field application and the development of vaccine-acaricide strategies deserve attention. Commercial expertise and co-investment will be essential to produce any registered product. In a number of ways, the MLA could play a constructive role in attracting a suitable commercial partner.

Executive summary

At the time of its commercial release in 1994, the TickGARD vaccine against *Boophilus microplus* was the first, and it remains the only, commercially available anti-parasite vaccine using a recombinant antigen. It was therefore highly novel, with a unique mode of action and recommendations for use that were very different from those of conventional acaricides. It is still available in a very limited way in Australia, as well as in Mexico and Cuba, but it must be counted a commercial failure. The reasons for this are complex and involve both technical and commercial factors.

The last decade and a half have seen an escalation of problems with chemical resistance. Since the original release of the vaccine, resistance has been reported in Australia or South America to three additional classes of chemical acaricide: the macrocyclic lactones, fipronil and the insect growth regulators. There is increasingly a realisation that any successful, sustainable program of parasite control is likely to require an integrated approach and a desire for "green" alternatives. All these factors make an improved anti-tick vaccine a desirable product.

Vaccine development is a complex, multi-stage process. Investment by the MLA could accelerate progress along the development pathway at a number of points. Currently, the greatest lack is in the availability of recombinant antigens of proven efficacy. A number of strategies can be used to identify these, as described in this report. Currently there is only one significant antigen discovery project in Australia and it is in early stage discovery with a restricted focus. Secondly, in the expectation that a vaccine superior to the previous TickGARD will require more than one antigen, the efficacy of antigen combinations using currently known or future antigens could be explored. The MLA could consider projects in either or both of these areas now. It is also in the identification of novel antigens or antigen combinations that the clearest intellectual property lies. Research issues downstream of antigen discovery are described and opportunities identified.

Loss of expertise in the general areas of tick biology and vaccine development has been considerable, particularly in the former. The availability of suitable experimental facilities has also declined. In any projects it funds, the MLA should consider the value of skills development and retention.

Development and delivery of a vaccine would benefit from the involvement of a major animal health company, both through financial support and technical and marketing expertise. This would be desirable early in vaccine development and essential in the later stages. However the gap between the results delivered by a typical research project and the technology package likely to attract investment by a major animal health company is always large, difficult to bridge and almost certainly increasing. The MLA could have a key role in helping to bridge that gap. For example, it is unlikely that a commercial company will be attracted by any antigen or antigen mixture that has not received some testing in cattle, extending even to limited "in field" evaluation. Support for such activities by the MLA would be both necessary and very productive.

While it is possible to map out the R&D that must be in place to convert a promising

antigen or antigen cocktail into a useful vaccine, it is much harder to be prescriptive about the steps that must be taken to create a technology package that will attract commercial co-investment. To assist in this however, this report includes descriptions by several major animal health companies of the desirable characteristics of a commercially attractive vaccine.

Early stage involvement of a commercial company could have benefits and disadvantages. The benefits are obvious; the disadvantages mostly relate to the risks of tying up of intellectual property at an early stage. On balance, early commitment of a strong partner is probably desirable. The MLA should consider ways in which this could be achieved, for example, through the Partners in Innovation program.

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1 Abbreviations

A. americanum	*Amblyomma americanum*
A. hebraeum	*Amblyomma hebraeum*
A. variegatum	*Amblyomma variegatum*
B. annulatus	*Boophilus annulatus*
B. decoloratus	*Boophilus decoloratus*
B. microplus	*Boophilus microplus*
H. longicornis	*Haemaphysalis longicornis*
H. anatolicum	*Hyalomma anatolicum*
H. dromedarii	*Hyalomma dromedarii*
I. scapularis	*Ixodes scapularis*
R. appendiculatus	*Rhipicephalus appendiculatus*
R. sanguineus	*Rhipicephalus sanguineus*

The species name *Boophilus microplus* is currently contested on the basis of gene sequence information. The proposed new name is *Rhipicephalus microplus* or *Rhipicephalus (Boophilus) microplus*. This is not yet universally accepted. In addition, structure of the genus *Rhipicephalus* is also contested. Further, new biological evidence strongly suggests that the Australian tick species is not *Boophilus microplus* anyway but another tick species. In such circumstances, this report simply uses the old name of *Boophilus microplus*, while recognising that this may be definitively revised in the near future.

2 Background

2.1 Vaccine development pipeline

Development of a commercial recombinant antiparasite vaccine is a slow, expensive, and complex process. Only a very small number have made it to extensive field trials, two to registration and only one to commercial release, namely the tick vaccine. The process is shown diagrammatically in Figure 1.

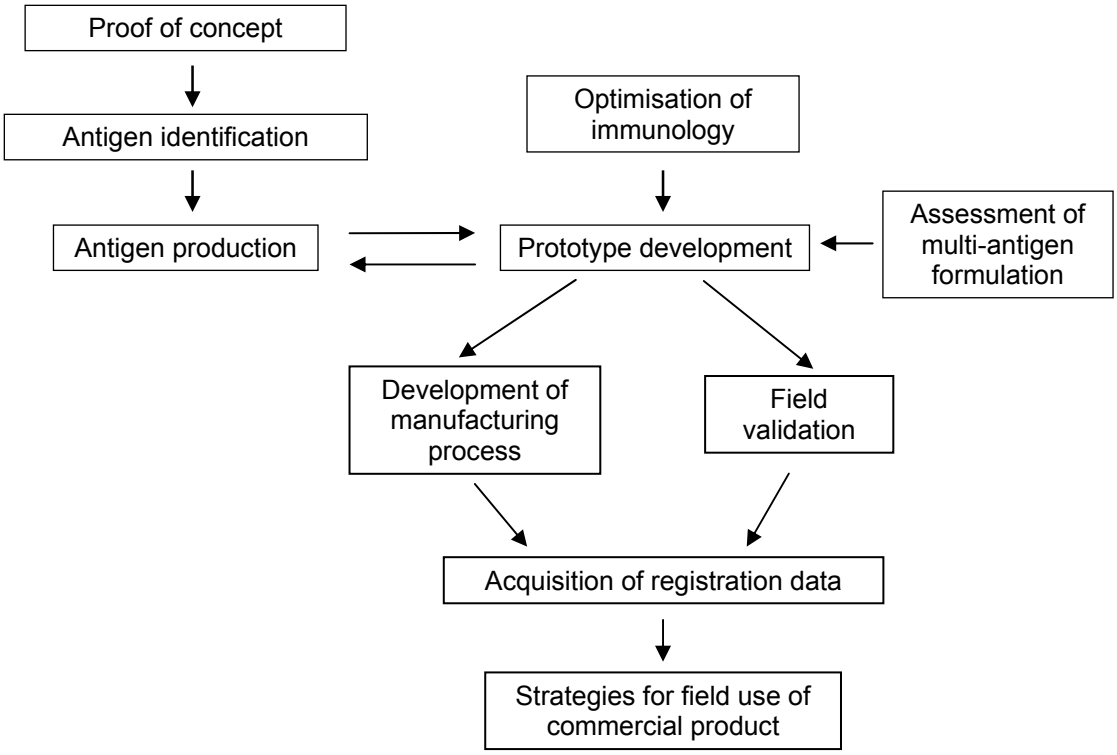


Figure 1. The vaccine development pipeline

Briefly, these steps involve the following:

Proof of concept
Usually an attempt is made to show that it is possible to artificially induce significant immunity to the target parasite through vaccination. Typically this is done with crude parasite material, or at best extracts of specific organs or partially purified extracts. Reverse vaccinology (see below) aims to circumvent this step and the process of antigen identification through the application of *in silico* analysis. This will be discussed later.

Antigen identification
Core to the production of the recombinant vaccine is the identification of an antigen or antigens capable of inducing efficacious immunity in their naturally occurring form. A number of strategies can be adopted, but this remains arguably the most difficult stage of vaccine development.

Antigen production

Cost effective production of an antigen for a tick vaccine demands that the antigen be produced as a recombinant protein. Basic methods for the production of recombinant proteins are now routine in many laboratories. For two reasons, the initial production of a recombinant protein is likely to be inextricably linked to the process of antigen identification:

(a) Even if a native antigen is effective, the recombinant may not be. Routine laboratory methods are unlikely to produce a correctly folded protein with glycosylation that is immunologically identical to that of a native tick protein, if that protein is naturally glycosylated. There are two consequences of this. Firstly, an ongoing comparison of native and various recombinant forms may be useful in optimising the activity of a commercially viable recombinant. Secondly, since success even then is far from guaranteed, a range of effective native antigens may need to be identified before enough are discovered that translate into effective recombinants.

(b) Some techniques for antigen identification screen recombinant proteins rather than the naturally-occurring “tick” forms.

Prototype development

Development of a prototype vaccine is a critical and complex process. Once the subsequent steps are entered on, namely (a) development and documentation of a commercial manufacturing process and (b) field validation of the vaccine, it becomes difficult and expensive to revise the nature of the prototype vaccine. There are likely to be three major inputs into the development of the prototype:

(a) Antigen production. It is likely that a number of different recombinant constructs will be assessed to find the most efficacious or at least the best combination of efficacy and cost of production.

(b Optimisation of the immunology. This may be nothing more than the assessment of a variety of commercially suitable adjuvant formulations, but the criteria that are used to assess these are critical.

(c) The assessment of multi-antigen formulations. Very few parasite antigens identified so far are sufficiently efficacious for a single antigen vaccine. It is commonly stated that greater efficacy can be achieved through multi-antigen mixtures but the experimental evidence for this is extremely limited.

Once a prototype vaccine is available, the full commercial development process can be initiated. This is likely to see two major streams of activity operating more or less in parallel.

Development of manufacturing process

This is a concern of the manufacturing company. It involves the development, optimisation and documentation of fermentation processes and downstream processing.

Field validation

Field validation will flow smoothly into the acquisition of registration data. The nature of the field validation that is undertaken is very much dependent on requirements of registration.

Acquisition of registration data
The purpose of this is clear.

Strategies for field use of commercial product
If a commercial product is released, there is likely to be support by the manufacturing agency or animal health company in its use. However, there is likely to be a role for further research in looking at the efficacy of various strategies for vaccine use. A specific example, as it applies to tick vaccines, is the way vaccine and acaricides can be used in an integrated program to achieve improved tick control in a cost effective way and with minimised chemical usage.

2.2 History of the TickGARD vaccine and its relatives: *TickGARD/ TickGARD Plus / Gavac / Gavac Plus*

2.2.1 Development of the vaccine and its commercial history

The development of the TickGARD vaccine began in 1980 with experiments conducted by CSIRO. Crude whole extract of partially engorged adult female ticks was used to vaccinate cattle. On subsequent infestation, there were variable but highly significant effects on engorgement percentages and egg-laying of ticks. It was also noted at the time that the ticks had a striking red colouration. Microscopic observation showed that this was due to the destruction of the gut, as shown by the massive leakage of bovine erythrocytes into the tick haemolymph.

The identification of the responsible antigen or antigens began in 1982 and led to the identification and partial amino acid sequencing of the Bm86 antigen in 1986. By this stage the development had become a collaboration between CSIRO and Biotechnology Australia / Biotech Australia, based in Sydney. Then, and for the duration of the collaboration, CSIRO was responsible not only for the initiation of the work, but also for all research into the native antigens; for the tick biology that underpinned the experimentation; for all initial evaluations of recombinant antigens and for the first field trials. Biotech Australia was responsible for the production of recombinant antigens and developed the capacity to conduct its own field trials, initially under the guidance of CSIRO. The development of the manufacturing process, field validation and the acquisition of the bulk of the registration data was the responsibility of Biotech Australia.

The first commercial vaccine, TickGARD, was released in 1994 and contained a recombinant antigen produced in *E. coli* in a standard adjuvant formulation. One year later, in 1995, TickGARD Plus was released. In this product, the recombinant *E. coli* antigen was replaced by a product from a *Pichia* expression system and the adjuvant formulation was changed to an in-house formulation. As a result, mean antibody titres in vaccinated herds were approximately twice as high as with TickGARD. This is particularly important for the efficacy of the vaccine. There is a good correlation between antibody titre and efficacy and so higher titre means simply higher and more sustained efficacy. Part of the increase in herd mean antibody titres was due to reduction in the number of cattle which showed very low antibody titres, i.e. the non-responder phenomenon was largely removed by change of adjuvant. The minimisation of the non-responder phenomenon was very important. As in many parasite populations, the distribution of tick numbers across hosts is highly skewed, a large proportion of the ticks being carried on a small proportion of the animals.

The chronology of vaccine development was:

1981	First proof of concept experiment with crude whole tick extract.
1982	Antigen identification begun.
1986	Key antigen (Bm86) purified, shown to be efficacious and partial amino acid sequence obtained.
1987	First trial with a recombinant antigen.
1989	First field trials.
1988-1992	Continual work to refine antigen production, adjuvant formulation. Efficacy testing proceeding in parallel.
1994	Product registered. Commercial release.
1995	TickGARD PLUS released as an improved vaccine.

Key patents were filed in 1985 and 1986. The patent containing Bm86 sequence information became publicly available in about 1988. A group of scientists at the Centro de Ingeniería Genética y Biotecnologia in Cuba, under the leadership of José de la Fuente, immediately began the cloning and expression of the Bm86 antigen in order to produce a vaccine, initially for Cuban use. Cuba, of course, does not recognise normal intellectual property rights. At the time it was believed that the enforcement of biotechnology patents in most South American countries would be either difficult or impossible.

In Australia initial uptake of the vaccine was very encouraging, as was feedback from producers that adopted it in the early years. Figure 2 shows sales of vaccine over the first four years after release. It must be remembered that at the time this was a very novel product with a novel mode of action, whose major impact was to reduce larval infestation on paddocks rather than directly control tick numbers on cattle.

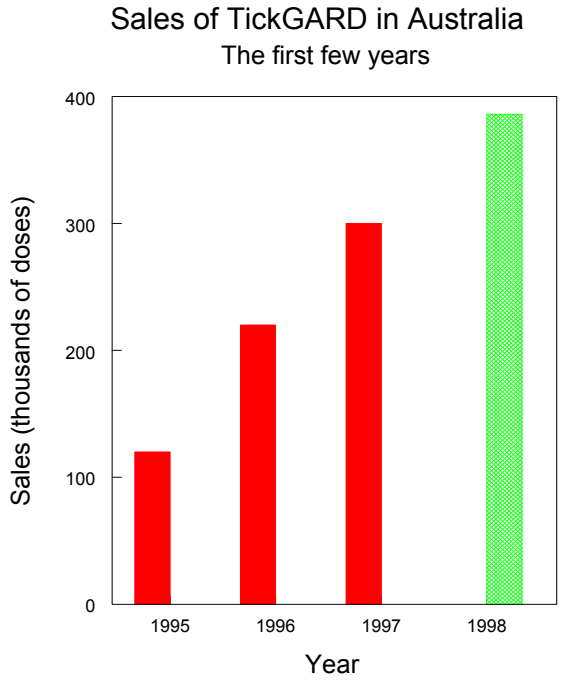


Figure 2. Sales of TickGARD vaccines in Australia. For 1998, sales figures are available only for January-July. The annual figure is an estimate based on the first six months of the year cf. 1997.

After 1998 a number of commercial factors impacted on the prospects for the vaccine in Australia. Initial reorganisations of Hoechst, by then the owner of Biotech Australia, led to significant changes in the company that were soon overwhelmed by the fragmentation of Hoechst AG, the parent company in Germany. Hoechst Animal Health became an independent company. The future of Biotech Australia seemed somewhat ambiguous as the company by then saw human pharmaceuticals rather than animal health products as key to its future. Subsequently, Hoechst Animal Health became part of Intervet and Biotech Australia ceased to be an operating entity. The licence to the IP for the tick vaccine passed to Intervet. The vaccine effectively disappeared from the market for some time. It is believed that within Intervet there were polarised positions on the desirable course of action with respect to the tick vaccine, some favouring the product and its ongoing development, others being opposed. Eventually it was reintroduced to the market in Australia after a delay of a number of years, at a higher price, and with changed recommendations of use that seemed to target it specifically at the dairy industry. Sales apparently did not rise above a fairly small fraction of those achieved by Hoechst. The late history of the vaccine was summarised by Playford (2005). Intervet terminated the manufacture and marketing of the vaccine. It also offered supply of the vaccine to the dairy farmers organisations on preferential terms and the vaccine continues to be available from that source, presumably from residual supplies. The IP for the tick vaccine reverted to CSIRO in its entirety. This IP has now expired, is expiring or has been discarded in every country except the USA, where it retains useful life.

From a strictly commercial point of view, the Australian market alone does not justify the production of a tick vaccine. The larger commercial markets are those in South America. The Australian vaccine was never marketed in South America, severely limiting its commercial appeal. A number of factors may have contributed to this:

- The commercial uncertainties and changes that affected the Australian situation must also have affected South America.
- The Cuban Institute for Biotechnology, via a Cuban commercial manufacturer, registered their vaccine in a number of South American countries and attempted to market it in Mexico, Colombia and probably Brazil for a while. It is difficult to tell how efficacious that vaccine was, though some trials would certainly suggest low efficacy. The commercial market for the Australian vaccine would certainly have been muddied by the Cuban version, Gavac.
- In both Australia and South America the limited field experience suggests that the vaccine is a very useful adjunct to tick control, but is unlikely to be a stand alone treatment in many circumstances, and then only with good management.
- It seems likely, despite questions around some of the experimental data (see later) that at least one tick isolate in Argentina is very little affected by the current vaccine. This again is likely to have confused the South American market, even when local tick isolates were vaccine susceptible.

For a discussion of some of these factors, see below.

2.2.2 Performance in Australia

The first field trials with the recombinant vaccine were conducted in 1990-1991. Herds of 15 cattle were maintained in separate paddocks that had been infested with equivalent numbers of tick larvae by earlier circulation of tick infested cattle through them at short intervals. Vaccinated cattle received a single priming dose of vaccine and one booster immediately before the expected spring rise. Results are shown in Figure 3.

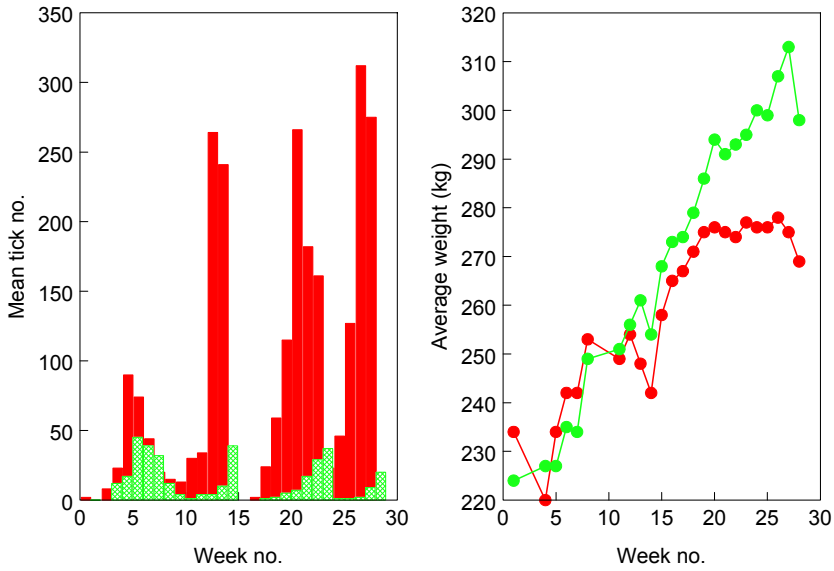


Figure 3. Field trial of a prototype vaccine.
The figures show the means for vaccinated cattle in green and unvaccinated controls in red.

By week 15, vaccinated cattle were in good condition and controlling the parasite infestation, but some animals in the control group were distressed and believed at risk of death. All cattle therefore received a single treatment of a short acting ivermectin. A number of conclusions were drawn from this experiment. Firstly, the differences between control and vaccinated groups were highly significant, with the vaccinates maintaining surprisingly good tick control until the end of the tick season, even in the absence of booster vaccinations. Though the graph shows numbers of engorged ticks rising towards the end, larval numbers were low. The trial was conducted at Amberley at Southeast Queensland, and the tick season was finishing. There was a significant benefit of vaccination on weight gain. This result cannot be translated uncritically into a more typical production situation. The trial was carried out under ideal conditions in that the vaccinated cattle were well separated from ticky, non-vaccinated animals; there were no cattle introductions and physical introduction of other ticks through contact with non-vaccinated cattle or by physical means was unlikely. Some variation in vaccine susceptibility of different tick isolates has been found in Australia and the isolate at Amberley was highly vaccine susceptible. On the other hand, the vaccine used in this experiment was a prototype with an antigen expressed in *E. coli* and an early adjuvant formulation. By comparison with a later TickGARD Plus, both antigen and particularly the adjuvant formulation would be expected to maintain higher and more persistent antibody response. That is, efficacy should be significantly better.

Extensive cattle trials, accompanied by serological measurement, showed that varying the time interval between primary and secondary vaccinations from two weeks to one year had little effect on the maximum antibody titre attained or the duration of antibody in circulation. Reduction of the inter-vaccination interval to less than two weeks tended

to reduce overall response, while the effect of extension of the interval to more than one year was not examined. In practice, this meant that, importantly, a priming vaccination could be given at any convenient time (say during winter). The timing of the secondary vaccination so that the early season generation of ticks was met by a good antibody titre however was highly desirable.

A number of post market release trials were carried out. For example, between September 1996 and June 1997 trials were performed on 26 sites covering 18 beef producing properties through the tick endemic regions of southern and central Queensland. The vaccine used was TickGARD Plus, the trials were run on commercial cattle properties and the measure of efficacy was the number of acaricide treatments which a producer chose to use to maintain a desired level of tick control on vaccinated compared with unvaccinated cattle or on vaccinated cattle compared with historical averages; that is, the use of the vaccine and tick control strategies was left substantially to the producer. Feedback was obtained at the end of the tick season. Vaccine booster injections were given at the producer's discretion. The main measure of vaccine efficacy was the decrease in the number of acaricide treatments on vaccinated animals. The results showed that all sites received a benefit ranging from 1-4 dips per booster vaccination with an average of 2.4. This saving was independent of the number of booster vaccinations actually given, cattle breed, dipping history or trial site location. One quarter of all sites achieved tick control without any chemical use, compared with an historical usage of 3-8 dips per tick season. Chemical free control was obtained most commonly (75% chemical free) in properties with a background of low dipping frequency, that is in dry cross-bred cattle. Ninety two percent of farmers reported that they would continue to use the product; non-use was in situations where there was low tick challenge, or the producer needed to maintain their current plunge dip anyway. Geometric mean antibody titres remained above a threshold level required for worthwhile effect on ticks on average for more than four months.

In another study, Jonsson *et al.* (2000) reported that vaccination of a dairy herd resulted in a 56% reduction in tick numbers in the field within a single tick generation, a 72% reduction in tick reproductive performance and an increase in liveweight gain of 18.6kg over a six month period. Further studies are lacking. Introduction of the vaccine to dairy farms occurred in a complex environment. The vaccine was first introduced as part of the dairy TickCON Program, a program of intensive acaricide plus vaccine usage originally intended to achieve on farm eradication. This occurred at a time when area wide tick eradication was being pursued as a matter of political priority. It is likely that the actual intent of the TickCON Program which was introduced on dairy farms in areas outside the formal tick eradication areas was not clearly conveyed. Initially it was seen as a commercial opportunity by Biotech Australia, that is as a small but significant market in a program promoted by the producers' own organisation. In retrospect however, it has been suggested that the introduction of the vaccine accompanied by intensive acaricide usage and without adequate justification for the overall program was arguably to the detriment of the market's perceptions of the vaccine itself. Nevertheless, it continues to be made available by the diary farmer organisation and anecdotal feedback from some producers at least is very positive.

2.2.3 International performance

Although in the early 1990s a number of pre-registration and field trials were carried out by Biotech Australia/Hoechst in several South American countries, very little of that assessment has been published. However, Hungerford *et al.* (1995) published a summary of several trials in Brazil. The aggregated data are shown below (Figure 4).

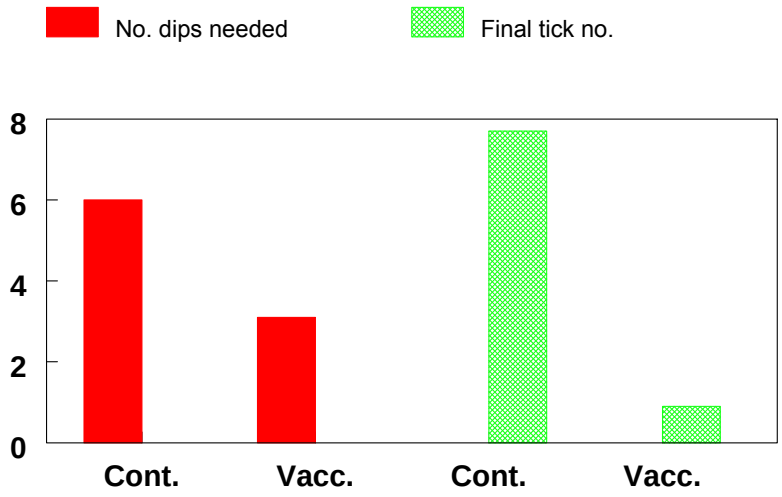


Figure 4. Aggregated results of field trials in Brazil.

Use of the Cuban vaccine in Cuba itself as well as Brazil and some other South American countries has been reported in more detail (Rodriguez *et al., 1*995a, b; de la Fuente *et al., 1*998, 1999; Valle 2004). Experience with the vaccine was broadly similar to that in Australia. Tick control could be achieved with good vaccine coverage and targeted, reduced acaricide application. An analysis of almost 600,000 dairy cattle between 1995 and 2003 in Cuba showed an 87% reduction in acaricide application with an overall 82% reduction in national acaricide consumption following introduction of the vaccine (Valle *et al., 2*004). Incidence of mortality due to babesiosis was also reduced. Interpretation of this data is complicated by the fact that introduction of the vaccine was accompanied by a change in recommended tick control strategy, from effectively total control with the concomitant heavy use of acaricides to a recommendation of threshold treatment. Nevertheless, much of the reduction in chemical usage can reasonably be attributed to the vaccine.

Experience in other South American countries has been varied, though there purely commercial factors have played a major role. It is summarised in de la Fuente *et al.* (2007a). Briefly, in Mexico, limited evidence suggests a 67% reduction in acaricide use on vaccinated cattle with an improved level of tick control. The state of Tamaulipas is sponsoring the use of the vaccine through government programmes. In Colombia and Brazil, various trials have suggested maintenance of tick control with reductions in acaricide use of 50% to 77% on vaccinated cattle. It was noted that problems with commercialisation in those countries have limited experience with long term vaccine application. There have been complicating technical factors as well. In

some regions of Mexico for example, there is a need to concurrently control *Amblyomma cajennense*, a tick against which the vaccine has no effect. A further complication is the occurrence in Argentina of a strain of tick apparently insensitive to the vaccine. This will be discussed below (Section 12.1).

2.3 Impact of genomics on future vacine development

2.3.1 The availability of tick-related genomic resources

The rapid development of genomic technologies will have an increasing impact on the identification of potential antigens. The availability of genomics resources and the current status of the technology were recently reviewed. (Jongejan *et al*., 2007). Briefly, EST (Expressed Sequence Tag) libraries, that is collections of sequences from genes that are actively expressed, are now available from a number of tick species and tissues. The Gene Index project at the Dana Faber Cancer Institute is a useful site for the tick data (http://compbio.dfci.harvard.edu/tgi/tgipage.html). Over 20,000 ESTs have been described from a normalised *B. microplus* library, derived from multiple tick stages and multiple acaricide susceptible and resistant strains (Guerrero *et al.,* 2005). There are collections of cDNA sequences for *H. longicornis* (Nakajima *et al.,* 2005) and the salivary gland of *Amblyomma variegatum* (Nene *et al.,* 2002). Targeted EST collections have been obtained from salivary gland cDNA libraries from *I. pacificus* (Francischetti *et al.,* 2005) and *I. scapularis* (Valenzuela *et al.,* 2002). Progress in definition of the "sialome" of ticks has been reviewed (Valenzuela 2004).

Far more ambitiously, sequencing of the genome of *I. scapularis* is underway (Hill & Wikel, 2005). The Ixodes Genome Project (IGP) was formed in 2004. Progress to date has been recently summarised (van Zee *et al*., 2007). Raw sequence data equivalent to five-fold genome coverage is already available. The task of assembly is proceeding and the process and challenges of that are briefly described in the van Zee paper. There are two related challenges to obtaining a full tick genome sequence. The first is the large size of the genomes, comparable to or even larger than mammalian genomes (Ullmann *et al.,* 2005; Palmer *et al.,* 1994). The genome of *Ixodes scapularis* is estimated to be 2.1x 10^9 bp and of *B. microplus* 7.1 x 10^9 bp or close to three times that of the human genome. The second challenge is the preponderance of highly repetitive sequences in those genomes. As Ullman *et al.* note "the most that can be predicted from the three tick genome measurements made to date (*Amblyomma americanum* had been examined previously) is that tick genomes will be large, highly variable in size and consist largely of moderately repetitive DNA." (Ullman *et al.,* 2005). Strategies that will be used to circumvent this problem in the case of *I. scapularis* are briefly described in the van Zee paper. Despite these challenges, as of mid-March 2008, an *I. scapularis* assembly consisting of 570,640 contigs in 369,495 scaffolds with a combined assembly length of 1.4 Gb was available, together with Blast resources:

http://iscapularis.vectorbase.org/GetData/Downloads/?&type=Genome&archive_status=current and http://iscapularis.vectorbase.org/Tools/BLAST/

In the paper by van Zee, a case is made there for the sequencing of additional tick genomes, and some of the pre-conditions for this to be successful are discussed. In fact, a proposal had been submitted for the sequencing of the *B. microplus* genome (Guerrero *et al.,* 2006) and assembly of useful resources, such as a BAC library and EST collections, has already occurred. The case was unsuccessful on the first attempt.

2.3.2 Genomics techniques

In parallel with genome sequence information, a number of genomics-related experimental approaches have had or will have substantial impact on vaccine development. These include RNAi, gene expression profiling and proteomics.

RNAi has become critical to the study of gene function. Experience with RNAi in a variety of parasite species has been mixed. Fortunately for those interested in tick biology, ticks have proved to be very amenable to manipulation by dsRNA, which has therefore quickly become a very useful experimental tool. A recent review by de la Fuente *et al*. (2007b) presents a possible mechanism of RNA interference in ticks, though experimental evidence is still sketchy. It then briefly describes the various techniques that have been used so far to deliver dsRNA before listing over thirty examples of the use of RNAi from the current tick literature. It concludes by briefly discussing the application of RNAi to the study of tick gene function, the characterisation of the tick-pathogen interaction and in screening for potential protective tick antigens.

The first published demonstration of the utility of RNAi in ticks showed the effect of down regulation of the histamine binding protein gene in *A. americanum* (Aljamali *et al.,* 2003). Success has since been achieved e.g. in silencing a cubilin-related proteinase in *H. longicornis* (Miyoshi *et al.,* 2004), the anticoagulant Salp 14 of *I. scapularis* (Narasimhan *et al.,* 2004) and synaptobrevin and cystatin (Karim *et al.,* 2005). The isac gene of *I. scapularis* codes for a novel anticomplement factor (Valenzuela *et al.,* 2000). It too has been successfully silenced (Soares *et al.,* 2005) as have a number of potential antigens in *I. scaplularis* (de la Fuente *et al.,* 2005) in each case with the demonstration of phenotypic effects. The most extensive use of RNAi in antigen identification has been by de la Fuente and co-workers and will be discussed later.

Gene expression profiling is potentially a useful technique for examining host responses to parasite infestation and various bovine microarrays have been available for some years now. Early work by CSIRO Livestock Industries using an in-house microarray has been published and work using the more extensive commercial arrays is continuing both there and in the Beef CRC. Although microarrays appear not to have been used by Brazilian scientists, they have carried out more limited but conceptually similar work by direct assay of the expression of a number of immune-related genes. Of more likely interest for vaccine development would be a gene expression in ticks in response to effect / ineffective immune responses by the bovine host. A first generation tick microarray has now been developed by Washington State University.

The application of proteomics to ticks has been slow which may be due in part to the lack of good genomic data: in the absence of that, or large catalogues of protein sequence information derived in more conventional ways, the interpretation of partial sequence information is difficult. A start has been made through the analysis of abundantly expressed proteins from unfed *B. microplus* larvae (Untalan *et al.,* 2005) and of tick salivary secretions (Madden, Sauer and Dillwith, 2002).

2.4 Current Australian research capacity

Until approximately the mid-1990s, Australia led the world in research on the control of *B. microplus* and as a result was also strong in the more general area of tick biology. That lead has been lost. It is debatable whether equivalent expertise has been developed anywhere in the world, but certainly individual scientists at the USDA and other organisations in the USA, in parts of South America, in particular Brazil, and in Japan are now contributing strongly to this field of research. Given the history of acaricide resistance, the ongoing commitment to tick research by major animal health companies is questionable. Meanwhile, in Australia, the history of the last decade or more gives concern about our ongoing ability to mount a credible research effort of any significant scale in the general area of tick control. It is therefore important to consider our current scientific expertise, availability of physical facilities and their use in ongoing vaccine development projects.

2.4.1 Expertise

Considerable physical and intellectual capacity is needed to develop a successful vaccine. Some of this is generic i.e. molecular biology, the production and purification of recombinant proteins, immunology and increasingly the fundamental experimental techniques of functional genomics. These areas of expertise are available in organisations like CSIRO, the University of Queensland and QDPI&F and elsewhere. CSIRO Livestock Industries has good in house proteomics capacity, likely to be important for one of the proven approaches to antigen identification (section 8.2 and 8.3).

More specific expertise in tick biology and the performance of field trials is problematic. In the final report of the MLA project AHW.054A (Playford, 2005) there was a list of scientists actively involved in tick research. The list was short and for very few of them was tick research a major commitment. In the intervening four years the list has become shorter. Scientists have left Australia, retired or shifted to other areas where research funding was available. Critically, Dr David Kemp, who was an important resource for general tick biology with great research experience in tick control, died in January 2007. This lack of expertise is a serious concern if future research into tick control or specifically vaccine development is contemplated.

Successful vaccine development requires inputs from multiple scientific disciplines, a good appreciation of commercial necessities, the ability to deal with intellectual property and legal requirements and some awareness of on-farm practice. The ability to draw these inputs together is not a trivial requirement and again would need to be taken into consideration in setting up a good vaccine program.

2.4.2 Facilities

One of the greatest constraints of vaccine development is the capacity to perform quantitative assays for antigens and prototype vaccines. Since most economically important livestock parasites are relatively host specific, experimentation with model or laboratory animals has very limited, if any relevance. Assays using livestock such as cattle are constrained by cost and frequently more importantly, by access to facilities where the required measurements can be carried out. Workers with the cattle tick are fortunate in that the effect of vaccination can be measured relatively easily and

quantitatively and by the standards of some parasites in a fairly short time period, e.g. four months.

The available assays for antigens include:

(a) Sheep as a model host.

Although *B. microplus* will complete its life cycle on sheep, our experience is that sheep make a poor host for larval ticks. However, they can be used as a good host for adults. Briefly, the process is to culture ticks in bulk on cattle through to the stage of nymphal engorgement. Nymphs are then removed individually with tweezers, allowed to moult in a humidified incubator and a known number of male and female ticks placed within aluminium rings glued to shaved skin on the back of a sheep. Engorgement proceeds as normal and engorgement percentage, mean engorgement weight and tick fecundity can be measured in a conventional way. For only one antigen, Bm86, is there information available on the relative efficacy of an antigen in sheep and cattle. There it seems that Bm86 is at least 10 times more efficacious in sheep than in cattle (de Rose *et al.,* 1999).

(b) Individually penned cattle.

Cattle are typically vaccinated, then housed in specially designed individual pens that allow the collection of ticks. They are infested regularly with a known number of tick larvae and the adult female ticks engorge approximately 3 weeks later, are collected on a daily basis, counted, weighed, and their fecundity estimated. This is the most quantitative assay available. Infestations are typically of 1,000 larvae a day, meaning that on tick naïve cattle, approximately 100 - 300 ticks per day will engorge. The assay preferably uses tick naïve animals to avoid the complication of naturally acquired immunity. The effect of naturally acquired immunity in this assay is simply to increase the variance in both control and vaccinated groups of cattle, meaning that group size must be increased to obtain a statistically significant result. In practice, for antigen screening, groups of three to four cattle were found to be adequate. As a variant on this procedure, large patches can be glued to the back or flanks of cattle and single larval infestations made within those patches. This process tends to be labour intensive and troublesome, but can be useful for specific experimental purposes. For example, it allows direct comparison of different tick isolates.

(c) Efficacy via side counts.

Tick infestation levels are routinely measured under more realistic field situations by counting the number of semi-engorged ticks (4 mm) on one side of an animal. This must be repeated on a number of occasions but with a skilled operator is able to give good quantitative or semi-quantitative data. If the cattle are given a standardised or at least equivalent larval challenge, this does allow estimation of vaccine efficacy under field conditions. Group sizes of course are larger. Statistical advice in the past has suggested that groups of 15-20 cattle are sufficient to detect a twofold difference in tick infestation levels.

For the identification of antigens and for most stages in vaccine development, the most useful assays are controlled pen challenges. The following summarises available facilities where such experiments can be carried out.

CSIRO Indooroopilly

This was the site of virtually all the experimentation that led to the development of the first tick vaccine. The experimental facilities were excellent, but were closed in the late

1990s. They are now inoperative and it would be difficult to return them to operational condition.

University of Queensland Vet Farm, Pinjarra Hills
Sixteen moated cattle pens are available.

QDPI&F, Yeerongpilly
Twenty two suitable pens are available. The current plan though is that animal work will finish on this site in August 2008.

University of Queensland Campus, Gatton
There is space for testing on 24 animals under PC2 conditions. Four moated tick pens are available.

Amberley
CSIRO operated a field station and two buildings of cattle tick pens for many years on a site near the Amberley Air Base. In the late 1990s these were relinquished and the lease was taken up by Bob Tozer and Flycam Pty. Ltd. Currently 24 individual cattle pens suitable for tick challenge and collection are available, together with limited surrounding pasture.

CSIRO Rockhampton
No cattle tick pens are available here but the site could be available for field experiments.

2.4.3 Current projects

QDPI&F, Dr. Ala Lew and co-workers
This is a project within the CRC for Beef Genetic Technologies (Beef CRC), funded by the CRC and a Queensland Smart State grant. The project is led by Dr. Ala Lew. Leading scientists in the research team are Ala Lew, Louise Jackson and Manuel Rodriguez who was recently appointed from the Cuban team that developed the GAVAC vaccine. Wayne Jorgensen provides scientific expertise in biological aspects of the project.

It was not part of the brief of this consultancy to review this project, since it has been reviewed within the CRC and its management is monitored by the CRC. The project team was however willing to be interviewed. The experiments that have been performed so far are very diverse: subtracted libraries of feeding cf. “frustrated” larvae; Holstein – Brahman contrasts in microarray experiments, paralleled with histological examination; serial sampling of Santa Gertrudis animals with high-low-middle resistance contrasts. Skin cells from Brahmans and Holsteins have been examined for genes differentially expressed in response to adult tick antigens. Such experiments are being used to compile lists of genes of potential interest. This is accompanied by bioinformatics analysis of the EST information collected by USDA, principally Dr. Felix Guerrero at Kerrville Texas. Bioinformatic approaches are a significant part of the project.

Although there was no explicit statement of the strategy for vaccine development, the experiments described have a clear focus on the tick-host interaction and in particular gene products that may be differentially expressed in response to that interaction. The assumption is presumably that these gene products are appropriate vaccine targets.

This is a testable hypothesis but clearly one that, particularly given the nature of acquired resistance to *B. microplus*, requires experimental validation.

As will be discussed briefly in section 8.1, bioinformatics is now an extremely useful tool for finding gene sequences with identified characteristics e.g. structural motifs or sequence conservation. The key to the utility of bioinformatics as a fundamental driver of vaccine development is a reliable paradigm for the selection of antigens. Whether this is available for ticks (or many organisms at all) is discussed in section 8.1.

Eventually though the list of candidate genes must be sorted through in vaccination trials or by using a good correlate of protection. A serious shortcoming of the QDPI&F project would appear to be the lack of provision for such trials. Funds are available for validation of a prototype vaccine in a field trial, potentially in 2010, but no or very limited funds for animal trials to bridge the substantial gap between a list of candidates and a prototype vaccine.

CSIRO Livestock Industries
The Wellcome Trust is currently funding (until the end of 2009) an international project aimed at examining the potential usefulness of anti-tick vaccines in the developing world, particularly sub-Saharan Africa. Collaborating laboratories are Utrecht University, The Netherlands; the University of Pretoria, South Africa; the University of Castilla la Mancha, Spain; CSIRO Livestock Industries, Australia; the Ecole Nationale de Médecine Vétérinaire, Sidi Thabet, Tunisia and the Veterinary Services Dept. in the Ministry of Food and Agriculture, Accra, Ghana. The African situation is of course far more challenging than Australia, with multiple overlapping tick species of veterinary importance and a number of tick borne diseases that cause very high mortality and for which vaccines are not available. The project is examining cross-protection of Bm86 homologues across a number of tick species in some detail. There is also a limited antigen discovery activity, focussing in South Africa on anti-haemostatic factors. The Australian component is small and directed at evaluating a very small number of potential vaccine candidates.

International projects
A small number of new antigens with claimed or potential activity against ticks are published in the scientific literature each year. Those identified to date are summarised in the tables that follow. The most active groups are José de la Fuente and his co-workers in Spain, the USA and Mexico; Professors Misao Onuma and Kozo Fujisaki in Japan and, occasionally, scientists in several South American countries, mostly Brazil. All of this is essentially university-based research and none of it amounts to a significant, consistent attempt to develop a practical vaccine. While insights and possibly valuable antigens may derive from these groups, the scale of activity is such that a commercial anti-tick vaccine seems unlikely to emerge from any of them in the near future.

2.5 Intellectual property

Some of the intellectual property relevant to tick vaccine development is listed in the Appendix. This material has been included principally to give a broad idea of the intellectual property landscape in the area. The limitations of this list are important. No attempt has been made to ensure that the list is comprehensive or to provide detailed analysis. Some intellectual property available in patent data bases which was known

to have lapsed has been deleted. It is likely that a number of the applications listed are no longer active. Neither has any attempt been made to gauge the validity of any particular patent application. Should it be necessary in future to obtain a thorough analysis of freedom to operate in the vaccine area, then that would need to be undertaken through a detailed, professional assessment.

A number of conclusions can be readily drawn:

- Patenting activity broadly reflects material that has appeared in the scientific literature. Quite a few of the antigens that are included in other tables in this report have also been patented.
- There has been very little activity by any major animal health company.
- Most patents attempt extremely broad claims, although this is not universal.
- A number of patents claim large numbers of unrelated gene sequences, while others claim degrees of sequence variation for their target molecule which are so large as to be virtually meaningless. No attempt has been made to follow these to check on their current status, or to see whether these claims have been finally allowed.
- Multiple tick species are frequently but not universally claimed.
- Importantly, in most circumstances there seems to be little or no biological data to back up the claim that a particular gene product could be useful as a vaccine antigen. In some cases there has been support for the claim in the scientific literature, but in many cases the claim is quite speculative.

In summary, there has been relatively little patenting activity in the area. There is little if anything of value in the patent literature that has not also appeared in the scientific literature. A number of patents could have nuisance value. A detailed analysis would seem to be inappropriate until there is a better idea of the nature of the antigens likely to come out of any Australian research effort.

3 Future R&D opportunities in vaccine development and application

3.1 What is the target product description?

Given the nature of the Australian regulatory system, it is very likely that the production of an anti-tick vaccine in commercial quantities will require the co-investment and collaboration of an animal health company with manufacturing capability. The involvement of such a company has the additional advantage of providing a strong route to market and probably promotion and technical support for the product. The principle (though not the only) aim of MLA-funded R&D should then be the development of a technology package likely to be attractive to such a company.

Of less immediate concern will be the need to satisfy Australian registration requirements. Though this will be principally the concern of a commercial company, some awareness of the likely requirements could be useful in setting research priorities even at an early stage.

3.1.1 Commercially desirable characteristics

To gauge the characteristics of a technology package likely to attract co-investment by a major animal health company, several such companies were contacted with the following questions:

- What, in their opinion, were the factors that had limited the adoption of earlier or existing vaccines?
- What would be the desirable, but realistic, characteristics a vaccine would need to have for it to be commercially attractive?

The contacts in each case were with likely key decision makers in their respective companies. Most were based overseas. From a global perspective the animal health companies are facing a number of pressures on profitability and the flow of new products, particularly when compared to human pharma: increasing costs of regulatory compliance, increasing times to market, increasing competition from generic drugs. The challenge is substantially greater for products targeted at food producing animals than for companion animals. Unsurprisingly therefore, one respondent pointed out that the burden of proof now required by a company was significantly higher than it had been in previous years. Comments from several major animal health were:

Opinion 1: The company is interested in a tick vaccine and I believe that what constitutes a viable vaccine profile has changed dramatically over time. Current thinking is that the vaccine should not be expected to act as a stand alone replacing chemotherapy. Rather an integrated approach which capitalises on the strengths of both a vaccine and chemotherapy is the target. Such an approach is "greener", should help to minimise and/or delay resistance, and is cost effective to the producer. As the animal health industry continues to consolidate, individual companies are better able to promote and sell integrated strategies than has been the case in the past. This should provide a better chance for a vaccine to succeed since an integrated approach somewhat gets away from the old ours is better than yours marketing, where the expectation was that the vaccine had to deliver the type of efficacy a drug did and do so immediately. A target profile might be something like this: 1.) a reasonable level of efficacy (70 to 85%) against adults and immature stages, 2.) > 90 reduction in reproductive measures, 3.) be effective in young animals (< 6 Months), and 4.) use fits with existing husbandry practices. Key to this profile is the use of a drug to get knockdown at or around vaccination and as needed thereafter.

From my understanding the Bm86 vaccine suffers from having previously being positioned primarily as a stand alone vaccine whether intentionally or as a product of inadequate education in the field. Existing preconceptions resulting from earlier field experiences will be a major hurdle to overcome but are not insurmountable. IP can be generated by developing cocktails or new delivery schemes. Clearly, the resistance situation adds value any alternative product.

Opinion 2.
A succession of four products could be considered:

- Reintroduction of a Bm86-based vaccine. MkI
- Bm86 plus added antigens for greater efficacy. MkII
- Bm86 plus antigens in a single dose formulation. MkIII

- Vaccine plus pharma formulation.

The first three are likely to be introduced sequentially, while the first and fourth could be introduced at the same time.

Opinion 3.
For livestock, where transmission of tick borne pathogens is not quite such an issue as it is for companion animals, vaccination is still considered within our company to be a potentially attractive "green" alternative to tick control provided the product profile is right:

1. Efficacy needs to be at the 70-80% level on a consistent basis under field conditions to satisfy Industry R&D, who understand the epidemiology and know that the modelling suggests that over time, this will be good enough. However, Marketing and other influential stakeholders want pharmaceutical (90%+) levels of efficacy and do not want to see ticks on the animal. This "disparity of views" has been a major barrier to many companies achieving internal consensus regarding tick vaccines.

2. One way the industry tries to be as lean and competitive as possible is to minimise the number of SKU's. In vaccines, this is a challenge as portfolio's often consist of multiple variants on a theme. For a tick vaccine to be attractive, SKU's must be kept to a minimum, ideally one! This means that we need cross protective efficacy against all commercially relevant tick types from a single vaccine at a consistent level of efficacy (point 1). This also means *no local products*, except in exceptional circumstances.

3. No crazy immunisation regimes - a 2 or maximum 3 dose course with at worst a six month booster would be essential, and an annual booster would be preferable, especially with an adjuvanted product. In the US, $10 on average is lost per beef carcass through the need to "trim" to remove injection site related damage. The more jabs, the bigger the problem, never mind the management issues.

4. The vaccine should not contain more than 3 recombinant antigens. We have a vaccine with 5 recombinant components for a porcine disease in our current portfolio and it is technically enormously challenging to produce (quality/consistency). We can only go so far with reassembling the tick before things get silly - a maximum of three antigens seems reasonable!!!

5. Ideally these antigens should be expressed in *E. coli*. Yeast/baculo/mammalian expressed antigens could be considered if it makes the difference between say 60% efficacy and 80% efficacy, but the higher the quality of the antigen, the lower the yield and the higher the cost of goods. There are examples of yeast or baculo expressed recombinant vaccines in farm animal vet medicine, but they are few and far between and probably marginal in terms of economic viability. What this means is that any R&D program should recognise this need up front and positively select antigens which are authentically expressed in *E. coli* rather than hoping the industry can sort out this superior conformation at a later date - often we cannot.

3.1.2 Registration requirements

Registration requirements for either a re-introduced, Bm86-based vaccine or a novel or improved vaccine would need to be discussed in detail with the APVMA by the commercialising entity. The following points are made for general information only.

The original manufacture of the TickGARD vaccine used both *E. coli* and *Pichia* fermentation systems. Unless the original master seed was still available, which may be unlikely, re-introduction of the vaccine would require new regulatory approval for the chemistry and manufacturing process for the "new" Bm86 antigen. Since the original master seed was constructed in the early 1990s, it may well be the case that an alternative antigen expression system would be chosen, though the expression levels originally achieved were exceptionally high. Efficacy would also need to be demonstrated, though this may well be considerably simpler than it was 15 years ago. The APVMA would be willing to consider a proposed trial design before any trial was conducted, to indicate whether the design would be acceptable for registration purposes.

The implications for research that might be co-funded by the MLA are (a) that a full registration process is likely to be required regardless of whether a Bm86-only vaccine or a multi-antigen vaccine were to be produced and (b) before any extensive trials were carried out, an assessment of their suitability as components of a registration package could and should be carried out.

3.2 Vaccine development: proof of concept

There are currently two approaches to the development of anti-tick vaccines, namely mimicry of naturally acquired immunity and the use of "concealed antigens".

3.2.1 Naturally acquired immunity or the use of secreted antigens

Cattle on exposure to *B. microplus* gradually acquire partial immunity. Initial infestation of tick naïve cattle leads to the engorgement of female adult ticks equivalent to 20-40% of the maximum theoretically possible. For example, a tick-naïve *Bos taurus* animal infested with 1000 larvae a day is likely to initially allow 100 – 200 adult female ticks to engorge. Over time the percentage return of engorged female ticks falls to typically 10% for many *Bos taurus* breeds and to perhaps 1-2% for purebred *Bos indicus*. *Bos indicus* animals, according to the older literature, not only acquire much stronger immunity, but also do so more rapidly. Resistance can become apparent even during the first infestation. There are also intra-breed differences in tick resistance. This was the basis for extensive experiments in breeding for tick resistance carried out, in part with support by the MLA, over a period of decades, principally in the 1960s – 1980s (Angus, 1996). A major component of this tick resistance, for *B. microplus* on *Bos taurus* cattle at least, is an acquired immediate hypersensitivity reaction to secreted tick allergens (Willadsen, 1980).

In principal, if these secreted antigens were identified, a vaccine might be developed on the basis of these antigens. Given that immunity is naturally acquired, it is legitimate to ask why one would bother to develop such a vaccine. The answer is twofold. Firstly it may be possible through, for example, the use of an appropriate adjuvant to produce a more efficacious immunological response to those same antigens than occurs during a natural infestation. It is a common feature of parasite

immunology the parasites are able to immunosuppress the host or divert the immune responses of a host towards the response that is not deleterious to the parasite itself. A synthetic vaccine may be able to reverse the trick. Secondly, vaccination would avoid the protracted tick exposure commonly necessary in *Bos taurus* cattle if they are to acquire their maximum resistance. This tick exposure will lead to concomitant production losses. The advantage of such a vaccine, in contrast to concealed antigens (see below), is that a vaccine based on secreted antigens could receive ongoing boosting during a natural infestation. That is, there may not be a requirement for repeated vaccinations. There are however potential disadvantages of such a vaccine e.g. the possibility of local inflammatory or allergic reactions in the bovine host.

For *B. microplus* there has been no report of successful vaccination that either mimics naturally acquired immunity or uses secreted antigens. That is, the proof of concept experiments have not yet been done or have been unsuccessful.

3.2.2 Concealed antigens

The term “concealed antigens” was used by Willadsen and Kemp (1988) and has since become a common one in the parasite vaccine literature. The basic idea is simple: parasites that feed on host blood or tissue fluid will inevitably ingest host immunoglobulin. In principal that ingested immunoglobulin could deliver an immune attack on the parasite. This does not occur naturally, simply because the ingested antibody does not have specificity for the parasite antigens with which it comes in contact, in the first instance antigenic molecules in the tick gut. The idea then was to vaccinate cattle with material from the tick gut or other internal organs and see whether antibody taken up with the blood meal was able to inflict damage on the tick. This was in fact found to be the case, and this observation became the basis for the development of the commercial tick vaccine. The antigen Bm86 is located on the microvilli of the tick gut digest cells. The advantage of the concealed antigen approach is that it greatly expands the repertoire of potential antigens. The disadvantage is that since these antigens are not exposed to the host’s immune system during a natural infestation, there is no boosting of the immunity by infection and so an effective vaccine response is only maintained through repeated vaccination.

In terms of proof of concept, this is unequivocally well established for B. microplus. The distinction between concealed antigens and secreted antigens can sometimes be blurred. In the practical sense however, the distinction is of value in that it bears on the very practical issue of whether a vaccine requires repeated boosting to maintain efficacy.

3.3 Vaccine development: antigen identification

The paucity of highly efficacious, recombinant protein antigens is the single major stumbling block to the development of an improved anti-tick vaccine.

Investment in further research into antigen identification and development could be considered in a number of areas:

(a) directed design: *in silico* approaches and educated guesses

(b) structured screening

(c) hybrid approaches

(d) further development of known *B. microplus* antigens

(e) use of homologues of antigens from other tick species

Each of these options will be considered in turn. There will therefore be some repetition in the information, since the same information can be informative in more than one experimental approach.

3.3.1 Directed design: *in silico* approaches and educated guesses

Knowledge of the likely characteristics of a protective antigen would be of enormous benefit to the cost effective development of a vaccine. The process of moving from tens of thousands of potential antigens, whether they be parasite proteins or genes, to a testable number of likely candidates relies on a series of filters. The filters may be pragmatic e.g. guided by actual vaccination trials, or "directed" in that they are built on the foundation of pre-existing knowledge. The latter, if successful, would clearly be more efficient.

The commercial development of acaricides / pesticides is an example, since the problems are somewhat analogous. At least two approaches have been used successfully in the past. The first has relied on the screening of chemical libraries against an insect or tick that is convenient for a bioassay. The second relies on the fact that the number of targets for the successful chemicals is quite small, much smaller than the number of active pesticides. Therefore a known or suspected target can be obtained in purified or semi-purified form and used as a basis for chemical screening, accompanied by a biochemical assay. Bioassay on the target pest / tick is then carried out on a smaller number of likely active chemicals.

Analogous approaches have been used or proposed for vaccine development. Directed design relies on the use of pre-existing knowledge to nominate or select antigens of potential interest. Such selection is followed by either isolation of the native antigen or its production as a recombinant protein followed by testing. At its simplest, this is the "Delphic" approach to vaccine development: compile a list of potential antigens based on biochemical and physiological understanding of the target parasite and experience with vaccination against other organisms, prioritise the list on the basis of a set of predetermined criteria, then test as many candidates as opportunity allows. The approach may be as reasonable as any other and have a fair chance of achieving success in a cost effective way.

The idea of prediction was taken to its extreme under the guise of "reverse vaccinology". "Vaccine development entered a new era when the genome of the first bacterium was sequenced in 1995. … possible to predict vaccine components by computer analysis without the need to grow the disease causing organism. For the first time after more than two centuries, Pasteur's principles of vaccine making were not necessary. The new method was named 'reverse vaccinology'. Today no vaccine project is approached without applying the concept of reverse vaccinology." (C.M. Fraser and R. Rappuoli (2005))

This is shown diagrammatically in Figure 5.

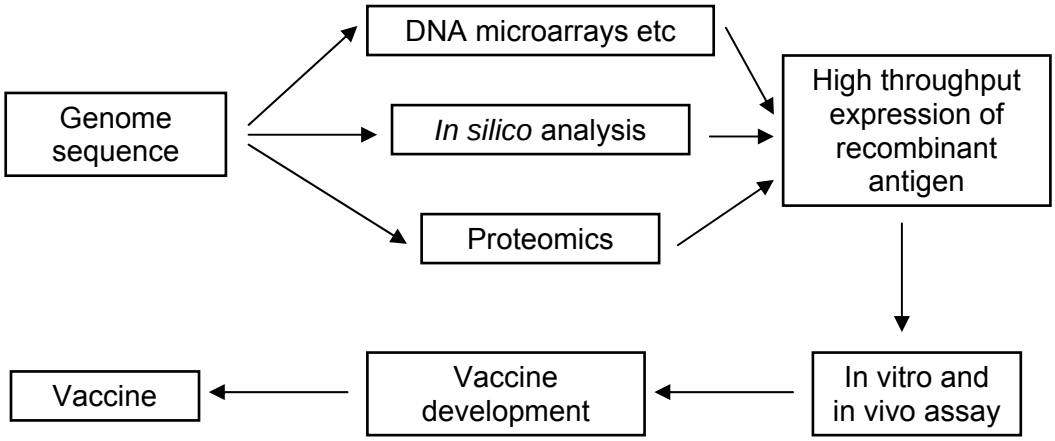


Figure 5. The reverse vaccinology approach to vaccine development.

The proposal is basically that using the genome sequence of the target organism as the foundation, a mixture of genomics technologies, including in particular a bioinformatic analysis. High throughput expression of the candidates is followed by some form of assay, proceeding to vaccine development. The claim is that this can produce a list of candidate antigens and subsequently a vaccine at a fraction of the effort required from more conventional approaches. Such processes have been attempted with a number of microbial pathogens, but the results to date have not been particularly encouraging. Central to reverse vaccinology must be a reliable paradigm for antigen selection. The genomics technologies, in particular *in silico* bioinformatics, can only be an efficient way of applying a scientific understanding of what is required for an effective antigen. It is doubtful that such knowledge is available for any parasite or for most bacteria, and certainly not for ticks. Nevertheless, as a long term objective, development of such predictive knowledge is highly desirable. It would make the development of vaccines much more efficient and cost effective.

With less hubris, it is valid to ask whether pre-existing knowledge of *B. microplus* or other tick species can guide the selection of probable antigens by class. If so that, combined with accumulating gene sequence information, would be a powerful tool for the production of putative antigens. The best that can be done to date is to look at the classes of antigens which have been investigated with positive outcomes. This is summarised in the following sections and Tables. The topic is discussed in more detail in Willadsen (2004) and in Willadsen (in press).

Salivary gland proteins and cement constituents as antigens

Older literature on tick feeding and immunology focused almost exclusively on the tick attachment site and the host-parasite interactions taking place there as well as on the constituents of the cement cone that is to a greater or lesser extent vital for the attachment of the tick to the skin of the host (for example, see Willadsen, 1980). Then, with the focus on the 'concealed antigen' approach to vaccination, this area of research seemed to become neglected for a number of years. This neglect is now being rectified. The biochemistry and functional genomics of tick salivary glands have been reviewed (Valenzuela, 2004) as have the function and physiology of the salivary gland (Bowman & Sauer, 2004). It is clear, thanks principally to work by Nuttall and colleagues, that the protein complement of the tick salivary gland shows individual

variation in addition to the expected species to species variation, as well as dynamic changes during the process of tick feeding. These differences are reflected in the antigenic profiles of the ticks (for example Wang & Nuttall, 1994; Lawrie & Nuttall, 2001). Clearly, the salivary gland and saliva are likely to be the vehicles by which factors such as immunomodulators, proteolytic and other hydrolytic enzymes, enzyme inhibitors and modifiers of haemostasis all pass from the tick to the host. These factors are likely to show considerable temporal variability. They will be discussed under separate headings.

There are other factors which do not fall into such groups, such as the structural components of cement. The salivary gland antigens identified to date are listed in Table 1.

Hydrolases and their inhibitors

The biochemical literature on hydrolytic enzymes is enormous, reflecting the ease with which they can be studied as much as their importance. The most studied group, the proteolytic enzymes, also possess a diverse and well characterized set of specific protein inhibitors. It is intuitively reasonable to think such enzymes would be key molecules in tick feeding and in the immune response. The role of hydrolases and their inhibitors in tick biology has been the subject of some speculation. A specific group of proteinases and inhibitors, namely those involved in haemostatic mechanisms, will be discussed separately.

From a vaccine perspective, serine proteinases for example are attractive because they are intimately involved not only in digestive processes but also in complement activation, blood coagulation and many aspects of the immune system. As such, it is possible that an immune attack on them could be deleterious to a tick (see, for example, Mulenga *et al.,* 2001, 2002). The disadvantage of proteinases as antigens is that they occur in very large numbers. For example, the *Drosophilia* genome codes for approximately 400 serine proteinases and one imagines that in many circumstances there will be a high level of functional redundancy, potentially making vaccination with one or a small subset of them ineffective.

Although there have been attempts to vaccinate against proteinases in other parasites, there has been little experimentation with ticks. Success with other parasites has been marginal. There has been speculation that a family of high molecular weight serine proteinase inhibitors, the serpins, could be target antigens (Mulenga *et al.,* 2001) and some evidence to support this. The tick hydrolases and hydrolase inhibitors used in vaccination trials so far are listed in tables 2 and 3.

Tick-induced host immunomodulation as a source of potential antigens

It is now well established that ticks modulate the immune system of their host in a variety of ways (see, for example, Barriga, 1999; Wikel, 1999; Wikel & Alarcon-Chaidez, 2001; Brossard & Wikel, 2004) while Titus, Bishop and Mejia (2006) catalogue the various immunomodulatory and haemostatic activities identified so far in hard ticks as well as other arthropod salivas. Many of these observations have been made with crude salivary gland extracts or, on occasion, through direct tick infestation of the host.

If the inhibition or diversion of the host's immune response is critical to tick survival, then it is possible that the tick molecules responsible for such manipulation could

themselves be vaccine targets. This has been suggested a number of times, but is still to be validated. The hypothesis here is either that the molecules are so critical to tick survival that their inhibition will lead to tick rejection or death, or that their inhibition by a vaccine-induced immunological response will ablate the parasite's own attempts at immune diversion, allowing the host to mount an effective rather than an ineffective immune response to the parasite. In any case, it is reasonable to assume that immunomodulatory molecules will be mostly secreted and so accessible to the host's immune system. Before these two related hypotheses can be explored, the nature of the parasite's immunomodulatory molecules must first be clarified. Fortunately, recent work by a number of groups has identified a range of candidates, affecting the immune system at various stages between antigen presentation and the effector response. These are listed in Willadsen (2004) and Willadsen (in press). The number of such molecules that have actually been tested in vaccination trials is very small (Table 4).

Tick anti-haemostatics as vaccine antigens.

Haemostatic mechanisms used by the tick to ensure the success of its blood feeding have been well reviewed (Mans & Neitz, 2004) and suggested as possible targets for immune intervention (Mulenga *et al.,* 2002). Protein anticoagulants, inhibitors of platelet aggregation such as apyrases and inhibitors of fibrinogen receptor function have all been described. Few if any have been evaluated in vaccination trials.

A recent paper by Maritz-Olivier *et al.,* (2007) reviews the diversity of tick anti-haemostatic molecules. No other group of tick proteins has received comparable attention. As the review makes clear, ticks have evolved a wide variety of agents capable of inhibiting host haemostatic mechanisms at a variety of points. Some 47 factors are listed, all being at least partially characterised. The review ends by briefly describing the potential usefulness of these molecules in anti-tick vaccines as well as in novel human therapeutics.

Gut and concealed antigens.

With Bm86 as the archetype, there has been much focus on vaccination with tick gut or gut membrane material, commonly with some success. The question naturally arises whether efficacy of an antigen is substantially or entirely due to its location, or whether some other functional or structural characteristics were also important. The question is important in practical terms, since it is much easier to identify a range of relatively abundant gut surface antigens than to identify a gut antigen of unknown abundance and specific if unknown functionality. This is a difficult question to answer conclusively, but the cumulative evidence is strong that that location alone is insufficient to identify a protective antigen. The evidence is:

- During the series of purification steps that led to Bm86, numerous fractions showed strong immunoreactivity with intact gut but little protective activity.
- Using a series of partially purified antigen mixtures, poor correlation was seen between anti-gut antibody, measured semi-quantitatively by immunofluorescence, and vaccine efficacy.
- Bm86 itself appears to be a minor membrane protein i.e. its effect is not related to abundance.

The known "concealed" antigens plus several others of miscellaneous type are listed in Table 5.

Missing molecules

What is obvious from Tables 1 to 4 is that they overwhelmingly consist of relatively small, soluble proteins that have a convenient biochemical assay or bioassay or that belong to families where there is sufficient sequence information for redundant PCR primers to give a good chance of detecting members of the relevant protein family. The list is very different from the list of targets for acaricides or other pesticides. Acaricide targets include one enzyme, membrane-bound acetylcholinesterase, but the rest are largely ion channels or transmembrane receptors. The same is broadly true for most targets of pharmaceutically active small molecules. There may be good reasons for pesticide and vaccine targets to be functionally and structurally different from the currently known tick antigens but the fact that they do not overlap at all needs to be considered critically.

In so far as anti-tick immunity relies on secreted molecules and the mimicking of immunity due to a naturally acquired infestation, then a focus on small, soluble protein molecules, particularly those to do with blood coagulation, proteolysis and so forth, as well as the likely constituents of tick cement, are obvious protein classes for examination. For vaccines that attempt the concealed antigen approach, the focus should logically be different. Clearly access of an antibody to the target molecule is critical for any vaccine, which is not the case for a relatively low molecular weight, probably hydrophobic chemical, where the ability to cross membrane barriers may be very different. Nevertheless, it is likely that the bias of experimental results in favour of easily assayable small and soluble proteins is largely a matter of experimental convenience, rather than a true reflection of their probable importance as vaccine candidates. The idea of using cell membrane receptors as vaccine antigens is an old one (see for example Sauer *et al.* (1994)) but has not yet been experimentally examined.

Summary

From the above discussion and the material in Tables 1 to 5, it is difficult to see that there is as yet a reliable paradigm for the selection of putative tick antigens from the plethora of possibilities embedded, for example, in tick genomic information. Development of such a paradigm should be a worthwhile long term objective but the limitations of our current knowledge in successfully predicting worthwhile antigens needs to be recognised.

Table 1. Presumed salivary antigens

Antigen	Species	Comments	Tested as:	Efficacy	Reference
P29	*H. longicornis*	Extracellular matrix-like protein	Recombinant	40-56%	Mulenga *et al.*, 1999
HL34		Tyrosine and proline rich. Induces by feeding	Recombinant	15-29%	Tsuda *et al.*, 2001
64P and 64 TRP constructs	*R. appendiculatus*	Salivary and "concealed" antigen; cross reactive between species	Recombinant	Up to 70% tested in guinea pigs against *I. ricinus, R. sanguineus*	Trimnell *et al.*, 2005 Labuda *et al.*, 2006
RIM 36	*R. appendiculatus*	Glycine and proline rich. Strong antibody response	Recombinant	None	Bishop *et al.*, 2002
Calreticulin	*A. americanum* and *B. microplus*	Secreted in saliva	Recombinant	Perhaps some effect in *A. americanum*. Poorly immunogenic (*B. microplus* version) in cattle	Jaworski *et al.*, 2002; Ferreira *et al.*, 2002)
P27/30	*H. longicornis*				You 2004, 2005

Table 2. Hydrolases

Antigen	Species	Comments	Tested as:	Efficacy	Reference
Bm91	*B. microplus*	Membrane-bound largely in salivary gland	Native and recombinant	50% as native protein; low or zero as recombinant but recombinant approx. doubles efficacy of Bm86 vaccine	Riding *et al.,* 1994 Jarmey *et al.*, 1995 Willadsen *et al.*, 1996
Aspartic proteinase precursor	*B. microplus*		Native		
5'-Nucleotidase	*B. microplus*	Membrane bound in Malpighian tubules	Recombinant	Uncertain; under test	Liyou, 1996
5'-Nucleotidase	*I. scapularis*	ELI vaccination	Recombinant		Almazan *et al.*, 2003
Esterase	*B. microplus*	Major allergen in naturally acquired immunity		Not tested	Unpublished

Table 3. Proteinase / hydrolase inhibitors

Antigen	Species	Comments	Tested as:	Efficacy	Reference
HLS 1	*H. longocornis*	Serpin	Recombinant	11-44%	Sugino *et al.*, 2003
HLS 2	*H. longicornis*	Serpin, in haemolymph, not saliva	Recombinant		Imamura *et al.*, 2005
	A. americanum and *B. microplus*				
RAS-1 and-2	*R. appendiculatus*	Serpins and concealed antigens; tested as recombinant protein	Recombinant	Nymphs 61%; Adults 28-43%	Imamura et al, 2006
Trypsin inhibitors	*B. microplus*	Tested as native antigens; effect possibly on larval development	Native	68%	Andreotti *et al.,* 1999 ; 2002

Table 4. Immune modulators

Antigen	Species	Comments	Tested as:	Efficacy	Reference
Histamine binding proteins	*Various species*	dsRNA in A. americanum induces aberrant behaviour / feeding		Not reported	Paesen *et al.*, 1999; 2000. Alijamali *et al*., 2003
Immunoglobulin binding proteins	*R. appendiculatus*			Slight or negative	Wang and Nuttall, 1999

Table 5. Other antigens

Antigen	Species	Comments	Tested as:	Efficacy	Reference
Bm86	*B. microplus*	Basis of the commercial tick vaccine	Native and various recombinant forms	Max. 90%	Numerous
Vitellin	*B. microplus*		Native and recombinant	Some efficacy as a native protein, none as a recombinant	Tellam *et al*., 2002
Voraxin	*Amblyomma hebraeum*	Two peptides create a factor that triggers female maturation	Recombinant	72%	Weiss and Kaufman 2004
BMA 7	*B. microplus*	Mucin-like protein	Native	50%	
4D8	*I. scapularis*	Relatively conserved across species. RNAi has a profound effect.	Recombinant	71% against adults	Almazán *et al.*, 2005b
4F8	*I. scapularis*				Almazán *et al.*, 2005a,b

3.3.2 Structured screening

Antigens identified by structured screening

In the absence of a good understanding of the characteristics of a protective antigen, it has often seemed logical to make as few assumptions as possible. Starting with material known to induce protection, one can screen it for antigens through fractionation to reduce the complexity, eventually, to a single antigen. Conceptually this is simple, though in practice it can be technically demanding. Such processes have been responsible for several of the antigens already mentioned, and they have contributed to the identification of many. Fractionation and screening as the major approach to antigen identification has been used for both proteins and genes.

Two screening processes have been described in the literature: protein and RNA-based screening. In the former, crude tick material is subjected to conventional protein purification procedures with the antigenic activity of partially purified protein fractions being assessed in vaccination and challenge trials. First and foremost such a process resulted in the identification of the Bm86 antigen, the basis of the commercial vaccines, as well as from *B. microplus* BMA7 and Bm91. In a second, conceptually similar approach, the screening is done at the level of RNA. This has been attempted only once for a tick. Almazán *et al.* (2003) used a cDNA library from *I. scapularis* to generate approximately 4,000 cDNA clones. These were then used for expression library immunisation in a series of mouse vaccinations followed by tick challenge. This led to the identification of a number of active pools of clones and a number of single clones which showed efficacy. One of these was the 4D8 antigen which has since been investigated in a variety of ways.

The group led by José de la Fuente has proposed a kind of hybrid of the predictive and screening approaches. They have suggested that RNAi could be generated and tested in a high throughput manner against the target tick species and that those target genes which resulted in a strong phenotype on suppression would be good target vaccine antigens. (de la Fuente *et al*., 2005)The evidence for the validity of this approach is that the 4D8 antigen, identified originally through ELI vaccination and confirmed as a recombinant protein, also shows a profound phenotype on down regulation with RNAi. However, suppression of Bm86 shows quite a weak phenotype though it is a good antigen, while the positive control used in many tick RNAi experiments is actin, where the phenotype is usually lethal. No one has yet suggested that actin will make a good vaccine antigen. The core assumption of the proposal is that a gene product central for the survival or normal functioning of the parasite will be a target vaccine antigen. Though superficially attractive, this is an unproven assumption.

3.3.3 Hybrid approaches

The directed design and structured screening approaches are commonly presented as mutually exclusive. The reality is that genomic information and high throughput experimental techniques have the potential to enormously enhance the structured screening approach. The protein fractionation techniques, for example, can often move quite efficiently from the thousands or tens of thousands of proteins in a crude extract to a mixture of, say, 100 potential antigens. The major time and expense are in refining that list of 100 to single, purified antigens with demonstrated activity and sufficient sequence information to allow recombinant expression to be initiated. Medium to high mass spectrometric sequencing, plus extensive EST or genomic information, coupled with bioinformatics, can greatly inform the identification and prioritisation of that list of 100, with great saving of time and cost.

3.3.4 Further development of known *B. microplus* antigens

Table 6 lists currently known *B. microplus* antigens, evaluated as either native or recombinant proteins. Bm91 received quite thorough evaluation and its potential to enhance the efficacy of a

Bm86 vaccine has been described in the literature. No evaluation of antigen B has ever been published. It was available as a native antigen in only low microgram amounts. Several trials with recombinant antigens failed to show consistent or striking effects.

Table 6. *B. microplus* antigens evaluated as native or recombinant proteins

Antigen	Status / Comments	Efficacy	Reference
Pro cathepsin	Evaluated as native protein		Da Silva Vaz *et al.,* 1998
Serine proteinase inhibitors	Native. Probably a mixture of multiple species	68%	Andreotti *et al.,* 2002
BMA7	Mucin-like, highly glycosylated protein	~ 50% as a native protein	McKenna *et al.,* 1998
Vitellin	Complex protein with glycosylation and bound lipid	Some efficacy as a native protein, none as a recombinant	Tellam *et al.,* 2002
Bm86	Evaluated as recombinant in multiple forms	Max. efficacy 90%	Willadsen *et al.,* 1995
Bm91 (carboxy-dipeptidase)	Evaluated as recombinant	~50% as a native protein; little efficacy as a recombinant but appears to enhance efficacy of Bm86 by ~ 2-fold	Willadsen *et al.,* 1996
5'-Nucleotidase		Uncertain	Liyou, 1996
Antigen B		Uncertain	Unpublished

There is little reason to think there is a lot of unexplored potential in these antigens.

3.3.5 Use of homologues of antigens from other tick species

Is it possible to extrapolate from experience with related vaccines or related parasite species on the assumption that an antigen that is effective in one species may be effective in another, or at least a homologue may be effective? As experimental evidence for this, occasional scientific publications over previous decades have suggested a degree of cross-protection in naturally acquired immunity between tick species, though the evidence is slim.

The most convincing evidence is that for the Bm86 antigen of *B. microplus* where varying levels of heterologous cross-protection between the *B. microplus* antigen and other tick species on a variety of hosts has already been demonstrated. This is summarised in Table 7.

Table 7. Other tick species: sequence conservation and efficacy of vaccination with *B. microplus* Bm86

Tick	% Sequence identity	Efficacy	Reference
B. microplus (Y)	100%	89%	Tellam *et al.,* 1992
B. decoloratus		70%	de Vos *et al.,* 2001
B. annulatus	97%($^{34}/_{35}$)	100%	Fragoso *et al.,* 1998;
			Pipano *et al.,* 2003
H. longicornis	37% ($^{l}/_{595}$)	?	Liao *et al*., 2007
R. sanguineus	67%($^{488}/_{63l}$)	?	Pickering *et al.,* unpublished
R. appendiculatus	78% ($^{114}/_{147}$)	~Zero	de Vos *et al.,* 2001
H. anatolicum	63% ($^{402}/_{632}$)	High	de Vos *et al.,* 2001
H. dromedarii	?	>98%	de Vos *et al.,* 2001
A. variegatum	?	0%	de Vos *et al.,* 2001

Numbers in brackets give the total number of amino acid identities as a fraction of the total number sequenced.

What this table shows is that this antigen shows a significant level of sequence conservation across species and that cross-protection is commonly observed, though not for all species. The fragmentary evidence also suggests that the level of cross-protection is not related in predictable fashion to the degree of sequence conservation. In some cases, protection is even stronger in a tick species than in the species of origin, while in others no protection or negligible protection is found even though species are relatively closely related.

Of course in the development of an improved vaccine against *B. microplus,* the question is reversed: are there highly efficacious antigens that have been identified in other tick species that might be useful candidates for *B. microplus*? The options for potential antigens are listed in Tables 1-5 above.

A feasible research approach would be to choose a selection of the more interesting or efficacious antigens from these lists, express the *B. microplus* homologues and evaluate them. While none of the antigens, as reported, have outstanding efficacy, the results with *B. microplus* might be different or, more realistically, the antigens may be useful for a dual- or multi-antigen vaccine (section 9.2).

3.3.6 Use of models

From the preceding discussion, three propositions can be drawn:

- The significant early stage bottleneck in vaccine production is in the identification of truly efficacious antigens.
- In turn, the bottleneck in antigen identification is the ability to screen numerous putative or potential antigens in an actual *in vivo* vaccination system.
- Antigens or homologues of antigens have a good probability of being efficacious in multiple tick host systems. That is, an antigen that is effective in one tick species is likely to be effective in another species.

If these three propositions are accepted, then there is an argument (for once!) for the use of a model tick host system that allows lower cost and higher throughput screening of antigens, whether native or recombinant, than is possible for *B. microplus* on cattle.

That is, the most efficient way of identifying novel antigens for *B. microplus* vaccines may not be to focus in the first instance on *B. microplus* itself, but on another tick species that allows the screening of larger numbers of potential antigens. The question then is which tick and which host could be used for such screening. As the literature cited above demonstrates, *Ixodes scapularis* has been used by American workers with mice and other smaller mammalian hosts. This is presumably because of convenience, vector capacity and increasingly the availability of genomic information and the resources that follow. In an Australian context, the use of an *Ixodes* species is possible but the phylogenetic distance between *Ixodes* and *B. microplus* is a disadvantage. It would be far better to use a tick species that is phylogenetically much closer to *B. microplus* than *Ixodes*. The obvious choice is *H. longicornis* on which Japanese workers have already done considerable research, also in terms of vaccine development, with rabbits as the chosen laboratory host.

Consideration could therefore be given to projects aimed at the identification of antigens from *H. longicornis* with rabbits as a model host followed by examination of the efficacy of homologues of those antigens in a *B. microplus* test system. Such projects could be suitable for PhD students.

3.4 Vaccine development: prototype vaccine development.

The process of prototype development, as before, involves principally the optimisation of the recombinant construct, the optimisation of the immunology and the assessment of multi-antigen formulations. Even at this stage, optimising the recombinant construct will preferably be done considering also the commercial viability for a final vaccine production process. This in turn means that the number of experimental systems that should be investigated is likely to be small. The process of doing so, though important, is predictable and so is not discussed further here.

3.4.1 Immunology

Immunology can be relevant to vaccine development in at least three ways: (a) antigen identification (b) enhancement of efficacy and (c) as a *de facto* measure of efficacy.

Antigen identification. If the aim of the vaccine development is to produce a vaccine that mimics naturally acquired immunity, and the immunological mechanisms which are responsible for that immunity are known with confidence, then in principle those immune responses could be used to screen for potential antigens. This could be a major route for antigen selection. In parasite immunology this idea has been much abused. There are too many cases in parasite immunology where Western blotting has been used to identify “antigens” with at best tenuous evidence that antibody is protective. Unless it is known that antibody is protective, there is an implied assumption that antibody responses will be a correlate of the actual protective responses, that is, that they will identify the same antigens. One of the more thorough recent examples of the use of a specific immune response to screen for antigens has been the use of a CD8+ T cell screen to look for antigens of *Theileria parva*. In the case of naturally acquired immunity to *B. microplus,* immediate hypersensitivity reactions seem to play a significant role in *B. taurus* cattle, and gene expression experiments carried out by CSIRO (Wang *et al*., 2007) and now the Beef CRC may have started to throw some light on potentially protective immune responses. However at the moment, it is hard to see that such knowledge is sufficiently secure to be a basis for antigen selection for a vaccine against this tick.

Enhancement of efficacy. There is considerable scientific literature examining immune responses to quite crude antigenic tick material such as whole salivary gland extract. It is again questionable that this contributed much to vaccine development and arguable that real examination of the immunological basis of protection is best undertaken once an effective single

antigen has been identified. At that stage however it becomes important, if possible, to identify the nature of the protective response to allow experimentation directed at enhancing that.

In the absence of such information, the best that can be done is to use the identified antigen in a number of different adjuvant formulations, preferably commercially suitable ones, and examine in parallel both efficacy against the tick and the nature of the induced immune responses. This may at least give a clue to the kind of immune response which must be elicited for protection and at the very least, contributes essential information towards the very pragmatic question of the best commercial formulation.

De facto measure of efficacy. Identifying an immunological correlate of protection can have enormous benefit in the registration process. An example is the development and registration of the TickGARD vaccines. Here it had been shown that there is a highly significant correlation between antibody titre and efficacy (Willadsen *et al.,* 1995). This meant that a lot of vaccine development could focus on the maximisation of the antibody response and its duration, with very substantial cost saving during the development process. That is, a variety of formulations could be evaluated without the necessity of doing parasite challenges on vaccinated cattle. The evidence for the role of antibody was strong enough so that in the registration process, serological evidence was used as a *de facto* measure of efficacy in some circumstances. This was particularly important as the number of treatment variables that had to be examined in that registration package was very large. In all, some 18,000 cattle were vaccinated before commercial release of the vaccine. So, for example, requirements to demonstrate that the vaccine would work in a range of cattle breeds, a range of age groups, and in a range of geographical areas, were satisfied in part by demonstrating that differences in antibody responses to a standard vaccination formulation across those variables were not significant.
To summarise, projects examining the immunological basis of protection and immune correlates of protection could well be desirable once effective recombinant antigens are available.

3.4.2 Multi-antigen formulations

It is very rare in immunoparasitology to find a single antigen that on its own is sufficient to deliver an efficacious vaccine. The best examples are from some cestode species. In the absence of such high efficacy, there is a core assumption, occasionally explicit but more frequently implicit, that the use of multi-antigen formulations will lead to the necessary minimum standard of efficacy. This is in reality a fundamental, but poorly acknowledged assumption of immunoparasitology (and arguably for other vaccines as well). In practice, there is astonishingly little scientific literature on the subject. In the case of *B. microplus* it has been shown that two recombinant antigens, Bm86 and an *E. coli* expressed version of Bm91, used together, have up to twice the efficacy of the Bm86 vaccine alone. Importantly, inclusion of the Bm91 antigen in a Bm86 formulation does not adversely affect the antibody responses of the more powerful Bm86 antigen (Willadsen *et al.*, 1996). There are no other examples of *B. microplus* and few for other parasites (Willadsen in press).

This is a worthwhile area for further research and one in which the MLA could play a role. The experiments to examine additivity or synergy in antigen formulations do not have the excitement of antigen discovery experiments, one probable reason for the fact that few are undertaken. More importantly, they can be relatively expensive experiments to undertake. Immune responses to a single antigen tend to be variable, and so the simplest experiment to examine the advantage of a two antigen mixture must attempt to show a statistically significant difference between three groups, cattle vaccinated with antigen A, cattle vaccinated with antigen B, and cattle vaccinated with antigens A + B combined. Given the variability of the immune response, the minimum group size is larger than if one is asking the question whether cattle vaccinated with a single protein are different from a control group, and so the logistics of the experiment are much more difficult. From past experience, an absolute minimum of 6 cattle per group, almost

certainly with replicates, will be required from each antigen combination. This is the kind of experimentation that is both essential for vaccine development and sufficiently demanding of resources to require co-investment by either an organisation like the MLA or a commercial company.

3.5 Vaccine development: development of manufacturing processes

It is reasonable to expect that an aminal health company investing in the manufacture of an anti-tick vaccine would assume responsibility for the development of a manufacturing process. There should be little or no role for MLA-funded research in this part of the vaccine pipeline. There are two qualifications however:

(a) If there was a compelling case for the development of a specific antigen production system, this might be a topic for research. An example would be development of an expression system capable of producing tick-like glycosylation on a recombinant antigen, using e.g. a tick cell line. This eventuality is unlikely in the short to medium term.

(b) It would be desirable that data from field trials contribute as much as possible to a future registration process. This may mean that the antigen production system, even if performed in a conventional molecular biology laboratory, should be aligned with a process that could be used commercially.

3.6 Vaccine development: field validation

The transition from controlled pen trials of the kind likely to be used heavily in the development of a prototype vaccine towards a vaccine with predictable performance in a typical production environment is likely to require a succession of field trials. These may range from relatively limited trials under reasonably controlled conditions to on farm producer and collaborator trials. In all of these the MLA could play a valuable role, as both a source for funding and active facilitator of the trials.

3.7 Acquisition of registration data

It is unlikely that the MLA would co-invest in activities targeted solely at the acquisition of data for registration of a vaccine. As before, some of the preliminary testing and evaluation of a prototype vaccine may contribute data useful in the formal registration process. It may therefore be worthwhile to be aware of likely registration requirements in designing such experiments.

3.8 Strategies for field use

Unless a vaccine has efficacy that significantly exceeds in efficacy and duration of effect any currently available acaricide (which is unlikely) then how it is used in the field is an important question. The TickGARD vaccine for example was released as part of three programs. One relied on a pragmatic combination with acaricide, designed to rely on producer knowledge and expectations, and appeared to be successful. A second, as part of the highly structured dairy TickCON program, must be accounted a failure. A third, recommending frequent vaccination, failed to make significant market impact. Clearly, strategies for use matter. The MLA could, in future, consider funding projects in this area though in the absence of a prototype vaccine with a probable product description, it is impossible to say just what experimental work would be justified. On past information however, it is worth commenting on two factors: the vaccine susceptibility of various tick isolates and the interaction between TickGARD and the macrocyclic lactones.

3.8.1 The effect of tick isolates

The existence and magnitude of variation in vaccine efficacy across tick isolates has been the subject to some experimental work as it has both practical and theoretical importance. It is discussed in more detail in Willadsen (2004). What follows is a brief summary.

The existence of such variation was discovered in the earliest days of the Australian tick vaccine development. Tick isolates from various parts of Australia as well as the acaricide reference strains used by Actest (the CSIRO acaricide evaluation service) and the QDPI were used in a variety of vaccination trials. In total approximately 20 isolates were examined. Significant variations in efficacy were found, from ~70% to ~90%, measured as total reproductive performance. Even at the lowest level of efficacy, the vaccine was able to achieve useful tick control when used appropriately. By coincidence, the most intensively used site during vaccine development and registration was at Calliope, and the resident tick population there had the lowest degree of vaccine susceptibility found in any Australian tick isolate.

The existence of such variation poses two questions of practical importance. Firstly, will the existence of a less-susceptible isolate of ticks allow for the rapid selection of resistance? Secondly, does variation in vaccine susceptibility correlate with sequence variation in Bm86, once again opening the doorway to selection for vaccine resistance?

Neither question can be answered unequivocally but current, fragmentary evidence on both is negative. After three years of intensive field trials at Calliope, ticks collected from paddocks on which vaccinated cattle had been grazed throughout showed no change in vaccine susceptibility compared with unselected ticks. In addition, Bm86 gene sequences were obtained from approximately 20 isolates. No correlation between sequence variation (up to about 3% across the molecule) and vaccine susceptibility could be seen.

Evidence from South America apparently contradicts this. The evidence is presented in Table 8.

Table 8. Relationship between vaccine efficacy and Bm86 sequence, relative to Yeerongpilly (Camcord) Bm86

***B. microplus* isolate**	**Sequence difference**	**Efficacy[a]**	**Reference**
Indooroopilly Y	0% (reference sequence)	89%	Tellam *et al.,* 1992
Tuxpan (Mexico)	5.7%($^{2}/_{35}$)	51%	Garcia-Garcia *et al.,* 1999
Mora (Mexico)	8.6%($^{3}/_{35}$)	58%	Garcia-Garcia *et al.,* 1999
Mexico	3.3%($^{22}/_{66o}$)	Same as Y	Cobon, 1997
Argentina A.	I.6%($^{10}/_{610}$ or $^{21}/_{610}$)	10%	Garcia-Garcia *et al.,* 2000
Camcord (Cuba)	0.2% ($^{21}/_{609}$)	84%	Montesino *et al.,* 1996
Columbia		81%	Patarroyo *et al.,* 2002

[a] Vaccinations for Indooroopilly, Mexico and Columbia trials used antigens based on the original Yeerongpilly sequence. The others were based on Camcord Bm86.

[b] The immunogen was a 43 residue peptide composed of three peptides from the original Bm86 sequence. The sequence difference in the challenge strain is unknown but likely to be similar to other South American strains.

The Argentina A isolate is reported to be essentially unaffected by a vaccine based on the Camcord gene sequence, which in turn is nearly identical to that of the Australian Yeerongpilly strain. An expressed Bm86 equivalent from Argentina A however was reasonably effective both

against Argentina A and other tick isolates. This “new” antigen was patented as Bm95. This seems convincing, but interpretation of the data has been made difficult by large inconsistencies in the sequences of Bm95s reported in various papers and the fact that the Bm95 sequence is nearly identical to sequences from Venezuelan and Mexican isolates, where no such differences in vaccine efficacy have been found. The question remains unresolved. Comparisons between series of independent and unrelated experiments is difficult; in measuring vaccine efficacy, differences in “tick biology” (unrelated to Bm86 gene sequence), antigen processing, vaccine formulation and stability and so forth may all play a role.

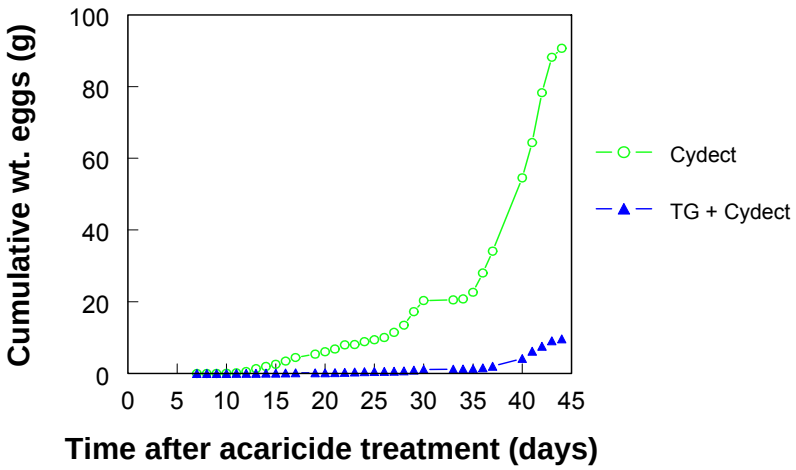
For current purposes, the question for the MLA to consider is whether isolate variability in vaccine susceptibility is a worthwhile researchable question. The answer is likely to be no, though it may well be part of an vaccine registration process.

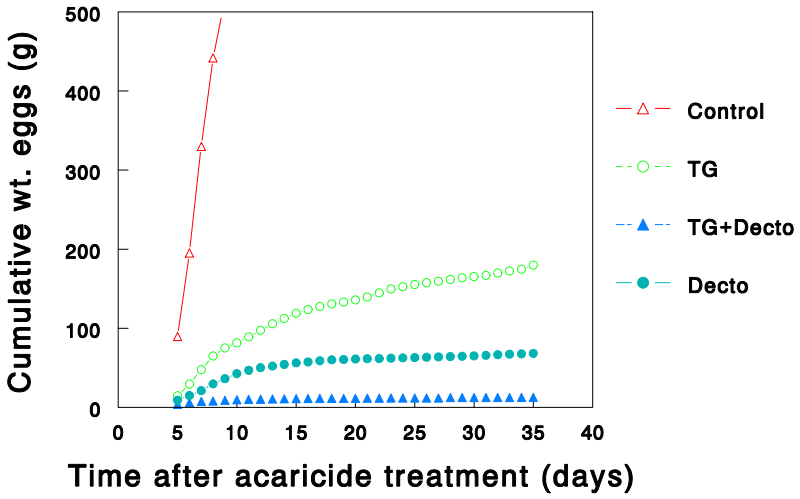
3.8.2 Joint vaccine – acaricide use

Joint use of the vaccine and acaricide can be seen in two ways:

a) As part of a simple integrated program with the use of the vaccine to suppress larval numbers on pasture and application of acaricide, though at reduced frequency, for the control of ticks if the numbers on cattle exceed a level acceptable to the individual producer. This is in fact the way the vaccine was marketed from the beginning and there is now quite extensive data showing that such a program can successfully reduce the frequency of application of acaricides by a factor of two or more, in the best instances eliminating acaricide usage entirely.

b) More interestingly, there could be a direct interaction between vaccine and acaricide if acaricide is applied to vaccinated cattle. One possible scenario comes from the fact that even in ticks surviving and engorging on vaccinated cattle, it is known that the permeability of the tick gut is greatly increased, perhaps by several orders of magnitude for serum proteins. It is therefore easy to imagine that particularly for an acaricide which is systemic, the effective dose that is delivered to a feeding tick might be significantly increased, potentially resulting in a synergistic effect. There is some evidence that this occurs for the macrocyclic lactones. Limited experimentation would suggest that synergism is not seen for either fipronil or Acatak. The following figures show data for cydectin and doramectin (Dectomax). The information has been published only in summary form (Kemp *et al.*, 1999).





These observations could usefully be confirmed and extended. They are potentially important. A combination of vaccine plus modern acaricide showing enhanced efficacy (more cost-effective use of an expensive macrocyclic lactone, or sustained efficacy at low levels of active chemical) may be one way to attract commercial interest in a vaccine.

3.9 Conclusions and recommendations

Capacity retention and capacity building. Success in developing a recombinant vaccine such as an anti-tick vaccine will only be achieved by a team with appropriate and diverse skills and with adequate infrastructure support. Even if these skills are available, it can be a challenge to assemble them into a workable team. Australia has adequate skills in generic science areas like molecular biology and immunology. Skills in tick biology and field experimentation have always been scarce and are becoming acutely so. Several decades ago, there were a number of scientists in Australia with a degree of familiarity with the cross-disciplinary challenge of vaccine development. That knowledge too is becoming less available, certainly outside the strictly commercial environment. Infrastructure e.g. for vaccine challenge trials has greatly reduced and is reducing further.

Recommendation:
1. In any projects the MLA supports, it should favour those that include capacity retention or development, particularly in "traditional" areas of tick biology. Specifically, PhD scholarships, with appropriate experimental support, may be a way to progress a few of the options below.

Antigen identification. Vaccine development is a stepwise process, though in the development phase, some steps can productively be run in parallel.

The current, major scientific limitation on the development of a new or improved tick vaccine is the lack of identified, efficacious antigens that can either replace or, more probably, supplement Bm86.

There is no guaranteed way of discovering effective antigens although a number of experimental approaches have been summarised in this report. Progress in the field as a whole has arguably

been limited by the frequent lack of either systematic approach or hypothesis formulation and testing. There is currently only one antigen discovery project of any size being carried out in Australia. On the information available when this report was written, this project is still very early stage and seems likely to progress no further than a list of candidate antigens that require experimental validation.

Recommendations:
2. The MLA could consider funding one or more antigen discovery projects. These projects should include a specific strategy for antigen evaluation and desirably be composed around clear and testable hypotheses. If this is done, then regardless of the success or failure of particular projects, progress towards intelligent vaccine design should be made, a worthwhile objective. Possible approaches have been summarised in the text above and in the Appendix.
3. When the current Beef CRC vaccine project is completed, new funding for the project should be conditional on a rigorous scientific review. Essentially, if there is to be an antigen evaluation process, then all potential antigens need to be considered, not just those arising from this project.
4. While there are currently no obvious candidate antigens of great interest, ongoing monitoring of the literature as it applies to all tick species is appropriate.

Prototype vaccine development. The potential of multi-antigen vaccines to demonstrate enhanced efficacy is almost entirely unexplored. This is a significant gap in our knowledge, not only for anti-tick vaccines but also for many others. In practical terms, it may be that a vaccine of desirable field efficacy is already "at hand" but unrecognised, in the antigens known from the literature.

Recommendation:
5. The MLA could look at the attractiveness of a project designed to examine this question. For example, it would be possible to evaluate four to six known antigens, or their homologues (if identified in other tick species) in combination with Bm86 or (potentially) each other. Obviously, depending on the rate of progress, antigens coming from a discovery process could be included in this.

Pre-registration field validation.
The stage in vaccine development at which a commercial animal health company may choose to become involved (i.e. invest some of its own financial resources) is unpredictable. It is quite likely however that most companies would be strongly attracted to even a limited field trial with recombinant antigens in a commercially acceptable formulation. Most companies may find such a trial a precondition to serious involvement.

Recommendation:
6. The MLA should stand ready to support such trials, if necessary. If there is reasonable evidence for efficacy of a prototype vaccine from even limited pen data, the MLA could consider funding a small field trial as an important component of a commercially attractive technology package.

Field application. The history of the market strategies adopted on the release of TickGARD and TickGARD Plus, and the consequences of those strategies, show that recommended strategies for use matter. Strategies for use would be an important part of any re-release if a vaccine is to achieve a desirable rate of uptake with producers and of field impact.

Recommendations:
7. Any field experimentation designed to provide advice to farmers on strategies for vaccine application should desirably use a registered vaccine product or, at least, a formulation with characteristics very close to those of a product to be registered. Once this stage is reached,

investment by the MLA into developing such strategies, and their subsequent extension, should be seriously considered.

It is worth noting that strategies an organisation like the MLA could recommend to its stakeholders may not be identical to the commercial recommendations for use derived from the registration process or as promoted by the commercial manufacturer.

8. An exception to the above that could be considered for research support is some systematic investigation of vaccine-acaricide synergies, in particular synergy with macrocyclic lactones. Such work might lead to the development of co-formulated products and so would need to be carried out at an earlier stage in the development process.

Commercial development.
Early involvement of a commercial (animal health) company in the co-development of a tick vaccine has potential advantages and disadvantages. The advantages include the potential for co-funding using a variety of mechanisms, ongoing exposure to commercial criteria and, particularly in the latter stages of development, access to in house expertise. Particularly if there is an interest in the commercial re-introduction of an anti-tick vaccine like TickGARD, early involvement of an animal health company would be essential. Potential disadvantages include complexity in the ownership and licensing of intellectual property and also the need to manage something as demanding as a vaccine development in a flexible and responsive way. The past history of the development of anti-parasite vaccines would suggest that rigid intellectual property and commercial arrangements were barriers to effective product development.

Recommendation:
9. The MLA could consider the desirability of and mechanisms for the early involvement of a major animal health company in vaccine development.

Clearly not all of these recommendations could be implemented in the near future, even with unlimited funds. Many are applicable to sequential steps in a process. In order of possible application:

Recommendations 2. and 5. could be considered in the near future, should the MLA decide to devote some funding to a vaccine initiative.

Recommendation 3 becomes relevant as the end of the CRC project approaches.

Recommendations 1 and 4 are appropriate to any ongoing project.

If any novel antigen is identified (as demonstrated by evidence of efficacy in tick challenge trials) then *Recommendation 6*. becomes appropriate.

Recommendation 9 could be explored at any time, from the present onwards. If an early stage commercial partner is obtained, research priorities will be greatly affected by negotiations with that partner. If a re-introduction of the existing vaccine is anticipated, then *Recommendations 7. and 8*. are immediately relevant. Otherwise, they must await the development of a prototype vaccine.

4 Appendix

Material in this appendix includes:

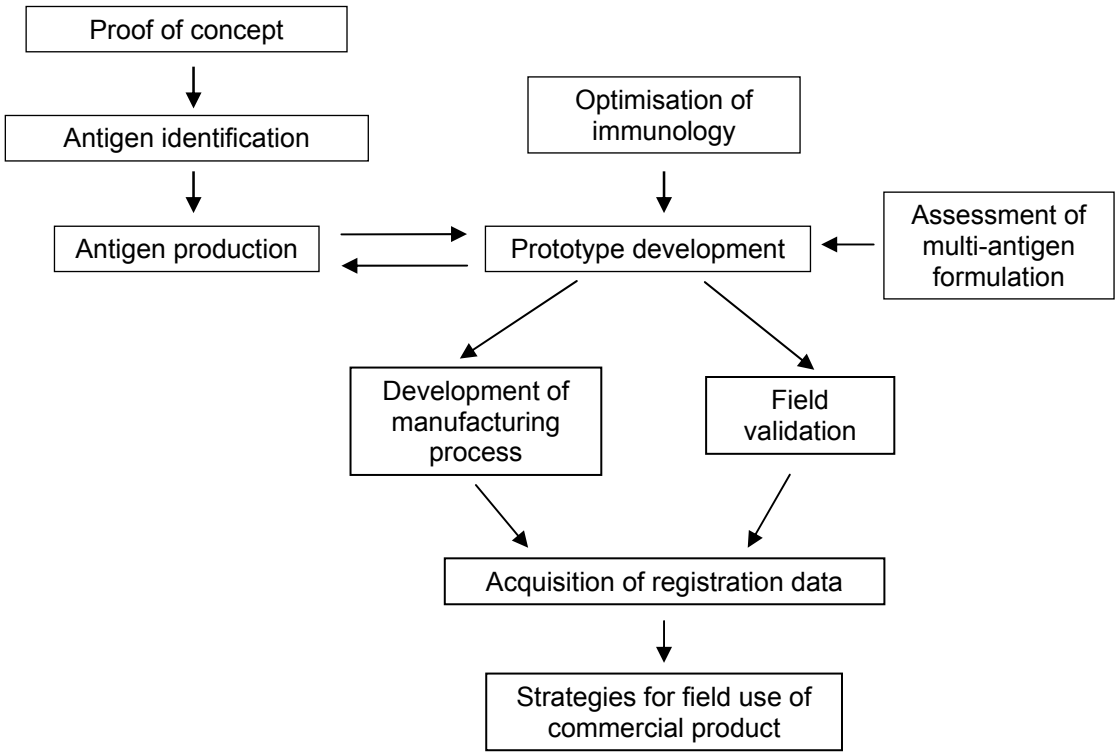
- What might a second generation anti-tick vaccine look like? A draft product description
- Summary of research opportunities
- Overview of patent literature

4.1 What might a second generation anti-tick vaccine look like?

	Old product (TickGARD Plus)	**New product**
Antigens/active ingredient	Bm86	Bm86 plus one other recombinant antigen
Target claim	Aid to the control of ticks on pasture	Minimum claim increased efficacy and duration of protection. Stronger claim possible.
Target market	Cattle, any age (post 3 months)"	Cattle, any age (post 3 months)"
Route of administration	Subcutaneous vaccination in neck	Subcutaneous vaccination in neck
Application scheme	One priming dose, timing not critical" Probably two boosters during tick season No priming vacc. in 2nd and later years (Tick season ~ 8 months)	One priming dose, timing not critical" Probably one booster during tick season No priming vacc. in 2nd and later years (Tick season ~ 8 months)
Technical distinction from competitive products		Zero withholding period Probable synergy with macrocyclic lactones Infrequent application Possible broader coverage of tick strains than old product. Suitable for integrated control program
Markets this product will enter		Australia / Central and South America Extensive beef production / grazing systems Use in pasture-fed dairy possible
Patent/licence protection		For Bm86 now only in the US. Novelty of formulation or novel antigen might be protected.

4.2 Summary of research opportunities

4.2.1 Vaccine development pipeline



4.2.2 Research opportunities

Stage in vaccine development pipeline	Activity / approach	Comments	Minimum resource requirement
Antigen identification	*In silico* prediction and educated guesses	A means to identify putative antigens. Under evaluation. Published track record with bacteria unimpressive; metazoan parasites more difficult.	Someone to do bioinformatics. Once putative antigen identified, see Testing of putative antigens below.
	Protein-based screening	Process used to identify Bm86 etc. Probably will not be attempted again.	A sustained program of perhaps 2 scientists plus technicians over three years minimum, plus animal facilities.
	ELI screening	Success with ELI in cattle has been low.	Resource requirements as for protein-based screening.
	RNAi screening	Makes assumption that an RNAi phenotype indicates probable antigen. Assumption poorly justified so far.	See next section
	Exploitation of known antigens	Makes the assumption that antigens known from other tick species are likely (as the *B. microplus* homologues) to be effective in *B. microplus*.	See next section.
Testing of putative antigens	Testing of putative antigens from: • *In silico* studies • Known antigens from other tick species • (Intelligent) guesswork • Expression of native antigens or antigens identified by ELI	A "best guess" list of putative antigens could be assembled and tested. To a limited degree underway in Wellcome Trust project. Could attempt e.g. 6-8 antigens in three years.	Each antigen for expression and testing 0.5 FTE (one technician, PhD student or scientist for six months) plus a minimum of 8 cattle (2 x 4), vaccination and tick challenge.
Prototype development / immunology	Comparison of formulations (adjuvants) for the prototype antigen combinations.	If the antibody response is the critical factor, serology is cheap and parasite challenge is probably not required. For salivary antigens the protective immune response is probably not known and so the situation is more complex and parasite	Minimum 10 cattle per group. Low cost. Perhaps 3 – 5 formulations to be tested.

Stage in vaccine development pipeline	Activity / approach	Comments	Minimum resource requirement
		challenge probably necessary.	
Prototype development / antigen combinations	Assume multi-antigen combinations (two antigens) will be needed for enhanced efficacy. This must be validated.	Assumption of increased efficacy through multi-antigen formulations critical but little tested. Reasonable evidence for Bm86 + Bm91. Needs to be validated for each new antigen combination.	Minimum 6 cattle per group, 24 per experiment (Control; antigens A, B and A+B). Parasite challenge essential.
Manufacturing process	Numerous issues, mostly relevant to a commercial manufacturer. Construction of a high expression vector a researchable issue.		
Field validation	Two objectives: • Demonstration of the superiority of New Vaccine over TickGARD or GAVAC in the field. • Satisfaction of registration requirements.	Depends largely on national requirements for registration.	"Superiority" demonstration probably requires a limited field experiment e.g. 10 + 10 cattle in a field situation, matched paddocks. Matching paddocks is difficult so replication probably desirable.
Activities not directly in the vaccine development pipeline			
Development of acaricide / vaccine combination strategies	Integrated strategies for farmers to use commercial acaricides plus vaccine in the most efficacious way.	There is evidence that (some or all?) macrocyclic lactones are strikingly more efficacious when used on vaccinated cattle. Benefits in lower cost / slower resistance development are possible. Optimal strategy needs to be developed. Access to (near) commercial vaccine desirable / necessary as desired outcome is a practical program.	
Re-creation of TickGARD	Patent now available, though with worthwhile protection only in the USA. No commercial product available	What is needed to bring such a vaccine back on to the market?	Depends heavily on national requirements.

4.3 Overview of patent literature

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
JP2007037464-A 03 Aug 2005	Tsuji, N. *et al.*	Novel polypeptide having galectin activity, useful in manufacture of pharmaceutical such as vaccine with respect to tick, for extermination of tick and treating or preventing tick mediated sexually transmitted disease.	A polypeptide having galectin activity …. useful in the manufacture of a pharmaceutical such as a vaccine with respect to tick	No evidence of efficacy
JP2006325497-A 27 May 2005	Fujisaki, K., Bordobatar, D. and Xuan, X.N.	Novel polypeptide having aspartic protease activity, useful as tick vaccine and for extermination of tick, and treating or preventing tick mediated sexually transmitted disease.	A polypeptide having aspartic protease activity and chosen from polypeptide having a fully defined 391 amino acid sequence given in the specification, …… and polypeptide having an amino acid sequence comprising 60% or more homology …. a tick vaccine, and for extermination of tick.	*H. longicornis*
JP2006246747-A 09 Mar 2005	Fujisaki, K. *et al.*	Novel leucine aminopeptidase polypeptide, useful for extermination of ticks and for the prevention or treatment of tick mediated sexually transmitted disease such as Piroplasma disease, Q fever, viral encephalitis.	Leucine aminopeptidase polypeptide, comprising a fully defined 527 amino acids sequence given in the specification …..or an amino acid sequence having 60% or more homology are useful in the preparation of a pharmaceutical, for extermination of ticks	*H. longicornis*
BR9903530-A 05 Aug 1999	Masuda, A. *et al.*	Anti-tick composition containing an aspartic protease consists of vaccine obtained from egg antigens for use on cattle.	The tick immuniser containing an aspartic protease comprises a vaccine obtained from the egg antigens. The vaccine can be used with or without other antigens, in cattle and other animals.	
BR200501904-A	Toro, F.P. and	*Boophilus microplus* tick control		No details at all

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
25 May 2004	Gutierrez, B.E.	vaccine incorporates tick larva integral protein antigen, preservative and additive.		
WO2005024022-A1; AU2004270780-A1; US2007275000-A1 10 Sep 2003	Kaufman, R. and Weiss, B.	Two new tick polypeptides that together have engorgement factor activity, and their encoding nucleic acids, useful in vaccines that inhibit tick feeding and development and prevent tick-borne disease.	Tick engorgement factor polypeptides and their encoding nucleic acids, are new. The vaccines are useful for preventing infection by a tick-borne pathogen or a tick-borne disease in a mammal, especially in a human. ……Vaccines including (fragments of) these proteins are useful against tick feeding and development, and offer protection against tick-borne disease.	The voraxin patent
WO2003093416-A2; US2004022795-A1; AU2003223767-A1 29 Apr 2002	de la Fuente, J. *et al.*	New cDNA molecule encoding an *Ixodes* associated antigenic polypeptide, useful in inducing an immune response against *Ixodes* species ticks, controlling tick infestations or preventing tick-borne transmission of viruses.	The cDNA molecule and vaccine are useful in eliciting antibody or inducing an immune response in mammals against Ixodes species ticks. Invention is a cDNA molecule, encoding an Ixodes associated antigenic polypeptide, comprises any of the 14 nucleotide sequences of 349, 2693, 1821, 697, 1221, 1942, 1428, 1847, 2475, 447, 1567, 704, 681 or 720 bp (ODD SEQ IN NOS:1-23, 24 and 25). All sequences are fully defined in the specification.	*I. scapularis.* Clearly attempts to patent a large number of sequences
WO2003030931-A; WO2003030931-A2 08 Oct 2001	Nuttall P.A. *et al.*	New vaccines comprising a 64p protein, useful against infectious diseases borne by blood-sucking ectoparasites, e.g. malaria, dengue fever, yellow fever, arboviral encephalitides, lymphatic filariasis, plague or Lyme disease	A vaccine composition against an infectious disease borne by a blood-feeding ectoparasite comprising as an active component a 64p protein having any of 8 65-224 residue amino acid sequences, given in the specification, or a fragment or homolog of the 64p protein or protein fragment that exhibits at least 50 % sequence identity with the protein or protein fragment, is new.	64P-associated patent
WO200180881-A; EP1283716-A; WO200180881-A1;	Trimnell A.R., Paesen G.C and Nuttall, P.A.	Tick cement protein for use in vaccine for immunizing an animal against blood feeding	A tick cement protein (TCP), or its fragment, or functional equivalent for use in vaccine … is useful as a component of a vaccine.	Patent of the 64TRP proteins

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
AU200150512 25 Apr 2000		ectoparasites and for the production of antibodies or antiserum specific to the protein.	The invention preferably contains one of the clone selected from clone 21, clone 33, CemA, clone 24, clone 68, clone 64 (64P) and clone I proteins or their fragments (64P is preferably 64trp cement protein, or their fragment or functional equivalent, as an active component and the fragment of 64trp cement protein includes 64trp1-6 or their functional equivalents).(I) further comprises a second active agent which is from a second immunogenic protein or protein fragment derived from a blood-feeding ectoparasite, and an adjuvant. Also disclosed are … a cocktail vaccine comprising two or more TCPs	
WO9924567-A; EP1029044-A; WO9924567-A1; AU9910471-A 12 Nov 1997	Paesen, G.C. and Nuttall, P.A.	Tissue cement proteins produced by blood-feeding ectoparasites and related polynucleotides	Tissue cement proteins (TCPs) produced by blood-feeding ectoparasites are new. The TCP, in a pharmaceutical composition, is useful for therapy, as a vaccine or vaccine component. The TCP itself is used to immunize an animal for production of such a vaccine. The tick TCPs provide a non-immunogenic tissue cement capable of bonding mammalian tissue with great strength. In detail, a TCP having a full-length amino acid sequence of 148 or 154 residues or containing any of the partial amino acid sequences (81, 90, 204, 114 or 65 residues)..The 81, 148, 90, 204, 114, 154 and 65 residue TCP proteins are translational products from clones 21, 33, cemA, 24, 68, 64 and 1. Products of clones 21, 33 and 68 resemble keratins. Clone 24 resembles glutenin. Clone 64 has partial similarity to collagen (i.e. the first 40 amino acids) and the remainder of the sequence resembles keratin. Clone 1	An attempt to broadly patent cement proteins for multiple uses. Contains 64P

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
			protein is similar to that of clone 64 and also resembles a cement protein of the reef-building polychaete *Pragmatopoma californica.*	
BR200004655-A 19 Sep 2000	Sadae Tanaka A. *et al.*	Antigen inhibiting ticks in animals consists of a *Boophilus microplus* trypsin antagonist of specific molecular weight	The antigen inhibiting ticks in animals comprises approximately 18 kDa molecular weight agent with its N-terminal portion determined by a sequence fully defined in the specification, for *Boophilus microplus* trypsin inhibition. Also claimed is a vaccine based on the antigen.	
WO200178770-A; WO200178770-A1; AU200155378-A	Jaworski, D.C., Barbour, A.B. and Barbour, A.G.	New macrophage migration inhibitory factor polypeptide and polynucleotide encoding the polypeptide, useful as a vaccine for inducing an immune response to tick, or for treating tumor or cell proliferative disease.	A substantially pure polypeptide comprising a sequence that is either at least 60-90% homologous to, comprising or having a fully defined 22 amino acid sequence useful for inducing immune response to a tick, or for treating a tumor or a cell proliferative disease. The MIF vaccine is useful for reducing or eliminating transmission of one or more infectious agents, in reducing fecundity of the female ticks that feed on the vaccinated animal or in reducing blood loss suffered by animals at risk of tick bites.	
WO200182957-A; EP1289545-A; WO200182957-A1; 04 May 2000	Patarroyo Salcedo J.H. *et al.*	New synthetic vaccine, for tick control, is formed by constructing *Boophilus microplus* 4912 or 7462 immunogen, constituted of continuous and defined sequence of amino acids found in different positions on protein Bm86.	A synthetic vaccine (I) against ticks formed by construction of synthetic *Boophilus microplus* 4912 (SBm4912) or SBm7462 immunogen, constituted of a continuous and defined sequence (S) of 43 amino acids found in different positions in protein Bm86 sequence, and its polymerization with cysteine in the N- and C-terminal of (S) is useful for producing immunity in bovines against ticks, and reduces their number and reproductive capacity (claimed). (I) is useful as a vaccine for tick control in bovines.	
JP2000083677-A		A gene encoding tick salivary gland antigen useful as a vaccine	A gene encoding a protein consisting of the amino acid sequence defined in the specification having an	

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
17 Sep 1998		for protecting animals from tick-carried infections	immunogen activity equivalent to tick salivary gland antigen; a tick-derived serine protease gene; a tick-derived cysteine protease gene; used as a vaccine for domestic animals such as cattle.	
BR9704150-A 26 Aug 1997	Masuda, A. *et al.*	Anti-tick vaccine for cows - comprises antigen isolated from bovine tick *Boophilus microplus*.	Anti-tick vaccine characterised by isolation of an antigen from the bovine tick, *Boophilus microplus*. The isolated antigen is a precursor of aspartic protease, more abundant in tick eggs but also present in haemolymph and the digestive system, where it is synthesised.	
WO200140469-A; WO200140469-A2; AU200119403-A; US2001046499-A1 03 Dec 1999	Kantor, F.S., Fikrig, E. and Das, S.	Novel *Ixodes scapularis* polypeptides for conferring tick immunity and for preventing the transmission of tick-borne pathogens.	An isolated, recombinant or synthetic DNA molecule (I) comprising a DNA sequence which encodes a tick polypeptide selected from fifteen novel *Ixodes scapularis* polypeptides, fully defined in the specification. Can be administered to a subject to confer tick immunity and therefore prevent infection by a tick-borne disease. Inhibiting coagulation factor Xa activity comprises administering to a subject a polypeptide selected from Salp14A, Salp9A or a fragment of these having Xa inhibiting activity, inhibiting histamine activity comprises administering a Salp25D polypeptide or its histamine binding fragment to a subject, and inhibiting or preventing an inflammatory response comprises administering a polypeptide selected from a Salp15, Salp25C, Salp13	Attempts to cover a lot of salivary proteins of different function. Limited vaccination data (in guinea pigs) given for two.
WO9849303-A; EP1017806-A 29 Apr 1997	Kantor, F.S. *et al.*	New DNA encoding epitope-containing polypeptides of I*xodes scapularis* used in vaccines to protect against tick infestation and tick-borne disease and for	New isolated, recombinant or synthetic DNA (I) comprises a sequence encoding an *Ixodes scapularis* polypeptide (II) that is(a) SP16 of 152 amino acids Also new are: (1) DNA (Ia) encoding 32, 28, 40 or 65 kDa I. scapularis polypeptides (IIa) (II) and (IIa)	

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
		diagnosis	contain protective epitopes, i.e. they induce an immune response that prevents or shortens tick attachment and feeding, so can be used to confer immunity against ticks.	
WO200077198-A; EP1187916-A; WO200077198-A2; AU200050558-A; 09 Jun 1999	Godfroid, E., Bollen, A. and Leboulle, G.	Characterization of genes induced in tick salivary glands during slow feeding phase of blood meal by cloning genes by forming subtractive library containing selectively induced mRNA during tick feeding phase.	A library of cDNAs which are induced in the salivary gland of a tick during the tick feeding phase … is useful for identifying genes induced during feeding. The therapeutic agent is used alone or in combination with an anti-tick vaccine to prevent the transmission of pathogens carried by the ticks. A tick salivary gland polynucleotide, preferably comprising sequences (S1)-(S28) or (S29) or its fragment; a tick salivary gland polypeptide encoded by (S1)-(S28) or (S29); A library of cDNAs which are induced during the slow-feeding phase of the blood meal.	
WO9406463-A1; AU9351032-A; 14 Sep 1992	Miller T. J. *et al.*		New immortalised tick cell lines and bovine T cell line used for the prodn. of antigens for vaccines and as a source of therapeutic and diagnostic agents. An immortalised tick cell line AGEC-1, ATCC CRL11083, its progeny and derivs. are claimed. Also claimed are (B) an immortalised tick cell line DGEC-1, ATCC CRL11084, its progeny and derivs., (C) an immortalised bovine T cell line Bpbl-T1, ATCC CRL-11120, its progeny and derivs. Antigens from the immortalised tick cells can be used for protecting against tick infestation or disease caused by a tick-borne pathogen, such as Lyme disease.	
AU8783331-B;	Cobon G.S. et al	Vaccine contg. tick antigens -	Novel immunogen comprises an antigen …. which is	

Patent no. and earliest priority date	Inventors	Description	Novelty	Comments
WO8803929-A; 27 Nov 1986		used for immunising a mammalian host, esp. cattle to induce immunity to tick infestation.	capable of damaging the plasma membrane of the gut cells of ticks feeding on the host. ….Also claimed is a polynucleotide sequence comprising a first polynucleotide sequence which acts as a coding sequence for amino acid sequences of the immunogen,	
ZA9302352-A; BR9300625-A 01 Apr 1993	Chaplen R.R. *et al.*	Production of tick particulate antigen in *Pichia pastoris* - useful as vaccine against *Boophilus microplus.*	Prodn. of a tick particulated antigen (I) in *Pichia pastoris* yeast comprises cloning the gene fragment coding for BM-86 Boophilus microplus antigen in the same yeast… is a powerful immunogen which may be used for immunoprophylaxis of bovine cattle against infestation by *B. microplus*.	
ZA9901320-A; BR9900780-A; MX9901724-A1 20 Feb 1998	Garcia Garcia J.C. *et al.*	BM95 Antigens of the cattle tick Boophilus microplus useful for preparing vaccine formulations.	An antigen (I) (designated Bm95) (comprising all or part of a defined 569 amino acid sequence given in the specification) from the cattle tick *Boophilus microplus* … is used to prepare vaccines to protect bovines against the cattle tick *Boophilus microplus*. The present invention provides a new antigen for preparing vaccines, that has a higher spectrum of action since it protects against all isolates of *Boophilus microplus*, including BM86 resistant isolates.	A sequence variant of Bm86

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