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Prepared by: A J (Bert) de Vos

Department of Employment, Economic Development and Innovation

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Meat & Livestock Australia Limited
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Theileria: Assess potential to develop a vaccine for *Theileria orientalis* infection

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Abstract

Bovine theileriosis caused by *Theileria orientalis* appears to be an emerging disease condition in eastern Australia and a sustainable method of control such as a vaccine is urgently needed. There are three variants of this parasite present in this country but only one is associated with disease. The purpose of this study was to determine from the literature how feasible the development of an effective subunit or live vaccine was likely to be. Not enough is known of the immune mechanisms of animals infected with *Theileria* for a subunit vaccine to be an option at the present time. However, development of a live vaccine based on one or more of the benign variants is worth considering even though significant differences exist between the variants. We recommend that the latter be progressed firstly by looking for evidence of cross-immunity between variants in small-scale pen and field trials. If there is cross-immunity, the technology exists in Australia to develop, register and produce a live vaccine.

Executive summary

The purpose of this study was to determine from the literature how feasible development of a vaccine for bovine theileriosis caused by *Theileria orientalis* is likely to be. This parasite is very common in Australia and is usually benign. However, major outbreaks of clinical disease have been reported in New South Wales during the past 5 years with mortality rates of up to 30% recorded in some herds.

A Theileriosis Working Group was formed to investigate this apparently emerging disease and rated, amongst others, the need to develop a vaccine as a priority for research. The exact cause of disease is still being investigated as part of MLA-funded project B.AHE.0038 but we already know there are three *Theileria* variants in Australia identified as 'Ikeda', 'Chitose' and 'Buffeli'. Indications are that only Ikeda is associated with disease outbreaks.

Two suggestions for consideration have been put forward regarding development of a theileriosis vaccine:

1. Infection with the benign Buffeli and/or Chitose variants of *Theileria* will provide adequate cross-protection against Ikeda and the technology is available in Australia to develop, test and produce a live vaccine based on one or both of these variants.
2. An effective recombinant or subunit vaccine can be developed.

In this scoping study, these suggestions are tested by addressing the following six questions.

How sound is the hypothesis that exposure to Chitose/Buffeli might render animals immune to Ikeda?

The hypothesis implies that there is cross-immunity between *T. orientalis* variants. While the variants cluster together on phylogenetic trees, there are major differences between them at immunological and molecular levels. These differences are based on analysis of, and responses to variable surface antigens but may also apply to actual infections. There is, however, no evidence of any studies done on infection-induced cross-immunity between the variants.

Live *Babesia bovis* parasites induce strong, lasting heterologous immunity while purified antigens tested only stimulate homologous strain immunity and a similar situation may well exist with *T. orientalis*. It will be possible to determine the presence or absence of cross-immunity following prior exposure to Buffeli and/or Chitose variants in small scale pen trials and through retrospective and prospective studies of relocated mobs of cattle in the field.

How difficult will it be to immunise animals to Chitose/Buffeli and then challenge them with Ikeda to test the hypothesis?

It will be easy to determine the potency, dosage, route of administration and shelf-life of a live vaccine by measuring the infectivity in vaccinated cattle with the use of molecular, serological and microscopical assays. There is currently no acceptable laboratory challenge model; and it will be necessary to assess vaccine safety and the level of protection in the field.

What are the risks of vaccinated animals being carriers and a source of virulent infection to naïve animals should they be moved into a "clean" area?

The risk is considered acceptable. Both Buffeli and Chitose variants result in life-long infections, are highly infective for tick vectors and most likely endemic wherever the vectors

are present and absent where they are not. Their inclusion in a vaccine is therefore unlikely to cause spread of disease. Because of a well developed host/parasite relationship, Buffeli is not associated with disease in cattle but there may be a potential risk of vaccine reactions in naïve, high risk classes of cattle if Chitose is included in the vaccine. In this event, certain precautionary measures will be indicated as is the case with tick fever vaccines.

How much is known of the complete genome sequences of Ikeda, Chitose and Buffeli variants?

Both Ikeda and Chitose variants have reportedly been sequenced but nothing has been published on this work.

What is the feasibility of developing a recombinant subunit Ikeda vaccine?

Development of a subunit vaccine that will protect cattle against Ikeda variant is not considered feasible, at least not in the short to medium term, irrespective of whether it is delivered as a protein in adjuvant or as a DNA construct. Because the harmful effects of *T. orientalis* are exerted mainly by the intra-erythrocytic piroplasms, the variable piroplasm surface proteins have received most of the attention in research on a subunit vaccine. Despite promising results of work done in the 1990s, progress over the past decade has been negligible. The slow progress being made in the development of vaccines against important vector-borne diseases such as malaria, East Coast fever and bovine babesiosis further suggests that it would be wise to focus on other means of control.

Is Biosecurity Queensland's Tick Fever Centre interested in developing and producing a Theileriosis vaccine as part of its suite of products?

If Buffeli and/or Chitose variants are shown to stimulate heterologous immunity against Ikeda variant, the Tick Fever Centre (TFC) of Biosecurity Queensland will be interested in developing and producing a live vaccine based on one or both of these variants. It is likely that the procedures and facilities used to produce tick fever vaccines can be applied to prepare a cryopreserved theileriosis vaccine without the need for additional resources. The vaccine will probably be provided as a multidose frozen concentrate to be reconstituted and mixed with diluent before use in the same way as frozen tick fever vaccine (Combavac).

No costings have been done but the cost of production will depend, amongst others, on the number of variants to be included in the vaccine. As TFC is a subsidised, Queensland Government-owned service provider, it does not have access to venture capital for new initiatives and funding will be needed for the development, evaluation and registration of the vaccine.

Conclusions

Development of a subunit vaccine is not feasible at the present time.

Development of a live vaccine is an option worth investigating. It is recommended that this be progressed through the following sequential steps:

1. Provide proof of the principle in controlled pen trials and by monitoring relocated cattle on affected properties retrospectively and prospectively ;
2. Develop, evaluate and register the vaccine.

Contents

1 Background

The purpose of this study is to assess the likelihood that an effective vaccine can be developed to prevent bovine theileriosis caused by *Theileria orientalis* in Australia. This parasite is very common[1, 2] and usually causes a benign infection[3]. However, widely distributed outbreaks of clinical theileriosis have been reported in eastern and central NSW during the past 5 years.[4, 5] Mortality rates of up to 30% have been recorded in some herds.[5]

Theileria was first detected in Australian cattle in 1910[1] and identified initially as *T. mutans*, a species native to Africa, then as *T. orientalis* and *T. sergenti.* [6] In 1984, Callow proposed to call it *T. buffeli* "until otherwise advised by the taxonomists".[3] It belongs to a group of closely related parasites referred to as the *Theileria orientalis/buffeli/sergenti* group.[7, 8]

Transmission studies in the 1980s showed that some *Theileria* isolates in Australia were transmissible by *Haemaphysalis longicornis*[9] while others were not.[10, 11] If vector specificity has any taxonomic significance, we therefore had at least two *Theileria* spp at that time following more than one introduction: [12]

1. With infected *H. longicornis,* a tick probably introduced from Japan more than a century ago.[13] It is a vector of *T. sergenti,* a recognised pathogen in that country.[14]
2. With infected cattle from Britain during the early days of European settlement.[15] *T. orientalis* is a common, non-pathogenic parasite in Europe and genomic studies have shown it and *T. buffeli* from Australia to be identical.[16] Vectors include *H. punctata* in Europe[10] and *H. bancrofti* in Australia, but not *H. longicornis.*[11]

Since then, international molecular studies based on sequences of a major piroplasm surface protein (MPSP) (p33) have shown that parasites in the *T. orientalis/buffeli/sergenti* group can be divided into 8 types or variants.[17] Three of these are now known to be present in Australia, namely Type 1 = "Chitose", Type 2 = "Ikeda" and Type 3 = "Buffeli". Sequencing of the small subunit ribosomal RNA (SSU rRNA) gene also allowed the group to be divided into 8 types identified alphabetically, including Type A = Chitose, Type B = Ikeda and with Buffeli close to Type C.[18] Another immunodominant surface protein (p23) also showed significant sequence diversities[19] with three identifiable variants named Ikeda, Chitose and Buffeli.[20].

Two workshops were held in 2009 to discuss this apparently emerging disease condition and led to the formation of a Theileriosis Working Group to investigate it further. According to the Working Group, diagnostic tools, identity of risk factors and control measures (chemotherapy and/or vaccine) were priorities for research. The exact cause is still being investigated as part of MLA-funded project B.AHE.0038 but we already know we have the three variants[21] and that only one (Ikeda) is consistently linked with disease outbreaks (Bailey unpublished 2011). When or how Ikeda entered Australia is unknown but the recent upsurge in outbreaks suggests it may be a recent introduction

Outbreaks are often associated with recent introductions of inland cattle to coastal districts in NSW or the introduction of coastal cattle to tableland properties.[5] Not all introductions lead to outbreaks and one large dairy farmer ("Farmer Brown") near Port Macquarie purchased cattle from different sources but only had trouble with those coming from "south of Sydney" and only if he introduced them between October and March. While we only hear of cattle movements when they are followed by clinical disease, there are probably many that do not have adverse consequences. What is particularly intriguing is the apparent situation where some cattle are affected clinically and others not – on the same property. There are many reasons why some cattle might mount more effective immune responses to Ikeda than others, including genotype (breed and even sire effects),[22, 23] the effect of environmental, nutritional and physiological stressors [7, 24, 25 26] and, obviously, the existence of active

immunity because of previous infection with this variant or, possibly, with a closely related one such as Buffeli or Chitose.

The hypotheses we put forward here are that:

1. Infection with the benign variants of *Theileria* (Buffeli and/or Chitose) will impart adequate cross-protection against Ikeda; and we have the technology in Australia to develop, test and produce a live vaccine based on one or both of these variants.
2. A recombinant or subunit vaccine can be prepared that will provide protection against the disease.

We will test these hypotheses based on what we can glean from the literature. If one is not rejected, we will recommend that projects be developed to firstly provide proof of the principle and, once that is done, to allow development of a vaccine.

The terminology used in the literature to identify *Theileria orientalis,* its variants and the disease caused by it is rather confusing. To simplify the nomenclature in this report, we will refer to the group collectively as *T. orientalis*, to the disease as 'Oriental Theileriosis' and to the variants by their names (Ikeda, Chitose and Buffeli), not by their alphanumeric identities. This may displease some, but our aim is to present what is known globally, and therefore local trade and other sensitivities are irrelevant. It may also offend purist taxonomists but this is not meant to be a taxonomic review either. We will draw analogies with *Theileria parva* (East Coast fever or ECF), but will ignore *Theileria annulata* even though there is an effective vaccine for it. [27] This *in vitro* produced *T. annulata* schizont vaccine is not relevant to *T. orientalis,* a parasite with few or no schizonts[7] that is very difficult to grow in culture.[28]

2 Project objectives

- Test the soundness of the hypothesis that exposure to Buffeli/Chitose might render animals immune to Ikeda
- Assess how difficult it will be to immunise animals to Buffeli/Chitose and then challenge them with Ikeda to test the hypothesis
- If the concept is shown to be viable, assess the risks of vaccinated animals being carriers and a source of virulent infection to naïve animals should they be moved into a "clean" area
- Determine how much is known of the complete genome sequences of Ikeda, Chitose and Buffeli variants
- Determine the feasibility of developing a recombinant subunit Ikeda vaccine
- Identify the interest of Biosecurity Queensland's Tick Fever Centre in developing and producing a Theileriosis vaccine as part of its suite of products

3 How sound is the hypothesis that exposure to Chitose/Buffeli might render animals immune to Ikeda?

3.1 Conclusion

The hypothesis that *T. orientalis* variants stimulate heterologous immunity is based on the fact that they cluster together on phylogenetic trees. In reality, there are significant immunological and molecular differences between them. These differences are based on immune responses to variable antigens and may well apply to actual infection with the respective variants as well. However, there is no conclusive evidence that anyone has actually looked closely at live parasite-induced cross-immunity between variants of *T. orientalis.* It is known that purified antigens of *Babesia bovis,* a cause of tick fever, only stimulate homologous strain immunity while live parasites induce strong, long-lasting heterologous immunity. A similar situation may exist with *T. orientalis* variants. It will be possible to determine the presence or absence of cross immunity following prior exposure to Buffeli and/or Chitose variants through limited proof of principle pen trials and through retrospective and prospective studies of relocated mobs of cattle in the field.

3.2 Literature review

3.2.1 *T. parva* – diversity amongst isolates

A key feature of the epidemiology of ECF is the diversity of parasite populations in the field.[29, 30,31] This is evident at both antigenic and molecular levels and commonly results in a lack of cross-protection between distinct isolates of the parasite.[32] Nonetheless, broad protection can be obtained with "infection and treatment" using relatively few strains, suggesting that antigenic variation might be limited,[29] especially in southern Africa.[31]

3.2.2 T. parva – live vaccines

An infection and treatment method of control using a live "vaccine" known as the "Muguga Cocktail" was developed about 40 years ago. It consists of three isolates to maximise the level of cross protection. Use of the cocktail was discouraged for decades (see section 5.2) but it is now registered in some of the 11 ECF endemic countries and promoted by Galvmed, a not-for-profit organisation funded by the Bill and Melinda Gates Foundation to protect livestock in developing countries.[33] The trivalent cocktail is cumbersome to make and some believe it is unnecessary as strains of *T. parva* exist (such as the well characterised Marikebuni strain) which provide adequate protection against heterologous challenge (McHardy pers comm. 2011). Morzaria and co-workers mentioned Marikebuni strain being used as a commercial "vaccine" in 2000. [34]

3.2.3 T. orientalis – diversity amongst isolates

A fair bit of work has been done on the taxonomic and immunologic relationships between the variants of *T. orientalis* we have in Australia. While we collectively lump them under the one species umbrella in this report and they cluster together on phylogenetic trees based on MPSP and 18S rRNA sequences, there is in fact wider sequence variation between them than between well defined species such as *T. parva* and *T. annulata.*[7, 35] Some authors consider the variants to belong to one species[8, 10] while others argue that they represent two species.[15, 16,36]

A phylogenetic tree based on rRNA gene sequences separates the variants into two groups with Buffeli (ex Australia) and Chitose (ex Japan) residing in one and Ikeda (ex Japan) in the other.[16] Chae *et al* (1999) argued that the first group was ancestral to, and clearly separated taxonomically from Ikeda (they called the latter Type B (*T. sergenti* Ikeda)) and concluded that it should be assigned an acceptable binomial seeing "*T. sergenti"* is invalid. [16, 37] A

phylogenetic tree based on p23 sequences[20] showed a similar relationship between the three variants.

Restriction enzyme site comparisons of PCR-amplified MPSP fragments also clearly discriminated Buffeli (ex Australia) from Ikeda (ex Japan).[36] Kawazu concluded that Ikeda (he called it *T. sergenti*) should be separated taxonomically from Buffeli. In ELISA and Western Blot analyses using native piroplasm antigens and sera from experimentally infected cattle, he could also discriminate immunologically between the two isolates.[36] Serological cross reactions between the isolates were low but some cross-reactive proteins were identified.[36] Kawazu did not include Chitose in this study.

3.2.4 T. orientalis –live vaccines

Immunisation by intentional infection of cattle with *T. orientalis* was first reported in Japan in 1962 (Minami *et al* 1981[26] quoting Ishihara *et al* 1962). From 1974 until 1979, a cryopreserved vaccine was used containing 2 x 10^8 infected red blood cells per dose. The vaccine "had an inhibitory effect on the clinical manifestation of theileriosis" but production has been prohibited since 1979 because of the risk of the vaccine spreading other diseases, including bovine leukaemia.[26] . Despite the prohibition a report published in 1992 mentioned the use of "some kind of vaccine" but wisely did not elaborate.[25] An attempt was also made to develop an attenuated whole blood vaccine in Korea in the early 1970s (Baek *et al* 1992 [38] quoting Suh 1972) but because of unidentified "constraints", attention shifted to use of purified immunogens.[38] The identity of the strains used in these vaccines is not known and if we decide to produce a live vaccine in Australia we will have to do it "from scratch".

The molecular and immunologic differences between variants mentioned above and the variant-specific immunity induced by recombinant antigens (see Section 7.2), have caused some (e.g. Sugimoto *pers comm*. 2011) to conclude that the hypothesis of protective heterologous immunity between variants has no scientific basis. However, we are not convinced. A rather similar situation exists with *Babesia bovis* where purified antigens only stimulate homologous immunity, yet live parasites induce immunity that is truly heterologous, even across national boundaries. We could not find evidence in the literature that anyone has looked at live parasite-induced cross-immunity between variants of *T. orientalis*. It will be possible to determine the presence or absence of cross-immunity following prior exposure to Buffeli and/or Chitose variants in relatively small-scale proof-of-principle pen trials and through retrospective and prospective studies of relocated mobs of cattle.

4 How difficult will it be to immunise animals to Chitose/Buffeli and then challenge them with Ikeda to test the hypothesis?

4.1 Conclusion

If it is decided to develop and produce a live vaccine based on Buffeli and/or Chitose variants, it will be easy to determine the potency, dosage, route of administration and shelf-life of the vaccine by measuring the infectivity in vaccinated cattle. It will also be possible to make preliminary observations on the vaccine's safety and efficacy but an acceptable laboratory challenge model is currently not available and it will be necessary to validate the safety and efficacy under field conditions. The Australian Pests and Veterinary Medicines Authority (APVMA) makes allowance for this in Guideline 47 (Data requirements and guidelines for registration of new veterinary immunobiological products).

4.2 Requirements

Guideline 47 of the APVMA applies. As per Part 8 (Efficacy and Target Animal Safety), the nature, degree, onset and duration of immunity are the main parameters of protection and all claims for efficacy, duration of protection and administration schedules are to be fully supported by data from specific laboratory trials and field studies. In the first instance efficacy and safety is to be demonstrated by experiments under laboratory conditions supplemented with data from field trials. However, "under some circumstances, such as where an acceptable laboratory challenge model is not available, field efficacy trials alone may be acceptable".

There is currently no suitable challenge model for theileriosis caused by *T. orientalis.* In a recent dose confirmation trial on the efficacy of buparvaquone against a mixed-variant isolate of *T. orientalis* at TFC, experimentally infected spleen-intact calves developed detectable, but very low, parasitaemias and no clinical evidence of disease. To increase the susceptibility of the calves, the trial was repeated, both with the mixed-variant isolate and with a pure Ikeda isolate, using splenectomised calves. While most calves developed marked parasitaemias, the clinical effect was limited with either isolate (Carter, unpublished 2011, Final Report of MLA project B.AHE 0048). Without the ability to reproduce the disease on a reliable basis, it will be very difficult to demonstrate protection against this specified disease in the laboratory.

APVMA makes allowance for this situation by accepting evidence from field trials. We recommend that the following be done if the work proceeds beyond providing proof of the principle that cross-immunity between variants exist:

- Make preliminary observations on safety in pen trials;
- Assess efficacy in controlled field trials in affected herds;
- Assess safety in field trials with different classes of animals in naïve herds.

As this will be a live blood vaccine, we anticipate that procedures and parameters used to produce and evaluate the efficacy and safety of tick fever vaccines at TFC will be applicable in this case as well:

- The same cryopreservation, storage and transport methods will be valid;
- 'Potency' equals infectivity with the presence of infection confirmed by microscopic, molecular and/or serological means;
- Persistence of infection (life-long in this case[7]) can be deemed to be an indication of persistence of immunity;
- Infectivity can be used to determine dosage and shelf-life;

- Safety can be determined using a combination of two parameters: anaemia and parasitaemia in blood;
- Efficacy after challenge can be assessed using the same two parameters.

To do this work, the following will be needed:

- APVMA approved GMP facilities to prepare a cryopreserved vaccine from the blood of calves (available at TFC);
- Naïve cattle and facilities for proof of principle trials (available at TFC and the Centre for Advanced Animal Science (CAAS), Gatton);
- Naïve cattle on co-operator properties for safety, potency, shelf-life and dosage trials (TFC pastures are infected, so are not suitable);
- At risk cattle on properties for field evaluation of vaccine efficacy (on co-operator properties with support from Livestock Health and Pest Authority (LHPA) and private veterinarians);
- A therapeutic agent to treat trial cattle (controls etc) which show clinical reactions;
- Sensitive, specific and affordable molecular and serological techniques capable of differentiating between the different variants (partly done).

5 What are the risks of vaccinated animals being carriers and a source of virulent infection to naïve animals should they be moved into a "clean" area?

5.1 Conclusion

The risk is considered manageable.

Infection with Buffeli variant results in life-long infection but, because of a well developed host/parasite relationship, it is not associated with disease in cattle. It has probably been present in Australia for as long as cattle have been here, is highly infective for native ticks and is expected to be present wherever the vectors are and absent where they are not. It is unlikely to cause or spread disease if used as vaccine.

Chitose variant also results in life-long infection and is highly infective for ticks. It was very likely introduced with the tick *Haemaphysalis longicornis* more than a century ago and is probably present wherever this tick and other native tick vectors are present. Its use in vaccine is therefore unlikely to result in spread of disease. It may not be totally avirulent and there is a potential risk of vaccine reactions if it is used on its own or in combination with Buffeli as vaccine, especially in naive, high risk classes of cattle. This situation will be no different to the one that already exists with the tick fever vaccines used in Queensland, so should not be seen as a disqualifying feature.

5.2 Likelihood of transmission

5.2.1 T. parva

Use of the "Muguga cocktail" as a live vaccine against ECF was mentioned in Section 3.2.2. It consists of three strains of which at least one results in a carrier state[29] and can be transmitted to unvaccinated cattle, thereby becoming incorporated into the resident parasite gene pool.[29, 39] A debate has raged for decades over the wisdom of using the cocktail in non-endemic areas (see for instance McKeever 2007 "Live immunisation against *Theileria parva*: containing or spreading the disease?")[29]. In the absence of alternatives, however, the cocktail is becoming more widely accepted in ECF endemic countries.[40]

5.2.2 Buffeli variant

All indications are that infection with Buffeli variant is life-long.[7] It is also highly infective for the indigenous tick species *H. bancrofti* and *H. humerosa,* but not the introduced *H. longicornis*[41-43] and, being identical with *T. orientalis* in Europe (see Section 1, Background), it has probably been in Australia for as long as cattle have been here. A serological survey conducted of *T. orientalis* (referred to as *T. buffeli* in the paper) in cattle in northern and coastal parts of Queensland[2] showed herd and animal seroprevalences to be 75% and 41% respectively. The test used was not variant specific and, while the cattle originated outside the *H. longicornis* infested area some would have been infected with Chitose (see below) or possibly even Ikeda. Even so, one can reasonably assume that Buffeli variant is present wherever the vectors *H. bancrofti* and *H. humerosa* are, and absent where they are not.

If this variant is to be used in vaccine, it will be infective for the tick vectors but movement of vaccinated cattle to a clean area will be inconsequential. If vectors are present, Buffeli will already be endemic and, if vectors are not present, then transmission will not occur.

5.2.3 Chitose variant

We assume infection with Chitose is also life-long as this is a feature of the group of parasites.[7] Nothing is known of its vectors in Australia but *H. longicornis* is the recognised vector in Japan[14] and is also present in New Zealand[44, 45] where Chitose is the only variant detected so far[44]. So it is reasonable to assume that this tick is the vector in New Zealand and, seeing they got the tick from Australia[46], that it is a vector here as well. However, preliminary results of a study of the *Theileria* variants in various parts of eastern Australia (MLA funded project B. AHE.0038) show Chitose has a wider distribution and much higher prevalence than Ikeda in Queensland. It was detected singly or as mixed infections with the other variants in 93% of positive animals compared with detection of Ikeda in only 14% of positive animals The latter is also (only?) transmitted by *H. longicornis*[14] and these results clearly suggest that Chitose has the good fortune of having other very effective vectors in this country as well. These vectors remain to be determined but *H. bancrofti* is one likely candidate. Chitose is also present in the USA in the apparent absence of *Haemaphysalis* spp. There, other tick genera including *Dermacentor* and *Amblyomma* are believed to be vectors.[47]

The situation if Chitose is included in vaccine is expected to be the same as that mentioned for Buffeli. For the same reasons, movement of vaccinated cattle to a clean area is likely to be inconsequential

5.3 Likelihood of live vaccine being a source of virulent infection

5.3.1 T. parva

The diversity of *T. parva* populations mentioned in Section 3.2.1 is also reflected in the virulence phenotype; some isolates are highly pathogenic and others very mild. The potential use of mild strains as vaccine has been considered but, in one review, “the possibility of reversion to virulence after tick passage” was considered to be “a serious counter consideration”.[48] The only example quoted related to work done in the 1960s[49] when a mild form of *T. parva* became virulent after passage through African buffalo. As *T. parva* is a natural parasite of the African buffalo (*Syncerus*)[31] with cattle (*Bos*) classical “wrong” hosts, this example is not applicable to *T. orientalis*, a parasite which, as discussed below, has probably had a very long association with cattle.

5.3.2 Buffeli variant

Identified as *T. orientalis* in Europe and as *T. buffeli* in Australia and elsewhere, this variant has a very wide distribution but we could find no evidence that it has been associated with disease anywhere. Even in splenectomised calves used in numerous transmission and chemosterilisation trials at TFC in the 1980s, clinical disease was not a feature. The association between *Bos taurus* and *T. orientalis* probably dates back to ancient, pre-domestication times so it comes as no surprise that there seems to be a very well adapted relationship between host and parasite.

The possibility of this variant becoming virulent as a result of mutation or genetic recombination cannot be ruled out but we believe it is extremely unlikely that this will happen. Much of western Queensland is free of *Theileria*[2] and naïve beef cattle are moved daily from these regions to endemic ones as part of routine breeding and finishing programs; yet we don’t see theileriosis even though many of them would certainly be exposed to Buffeli. We expect the same outcome if naïve cattle are exposed to vaccinated ones in the presence of vectors, while nothing should happen if vectors are absent.

5.3.3 Chitose variant

Very little is known of the pathogenicity of Chitose variant. It is one of five variants in Japan[18-20] With the majority of animals showing mixed infections,[20, 50] the epidemiology of theileriosis in that country is very complex. It is also present in Korea,[17] Thailand,[51] China,[19] Russia[17] and Turkey.[52] Most of the field reports of clinical disease in Japan and Korea[20, 53] deal with mixed infections of at least Ikeda and Chitose. There is no evidence in the literature that anyone has experimentally looked at the pathogenicity of Chitose *per se.*

Recently, however, an outbreak of haemolytic anaemia associated with *T. orientalis* was reported in New Zealand[44] where, based on rRNA sequence data, the parasite showed the greatest similarity to Chitose. The outbreak occurred in a herd of 87 dairy cows and heifers relocated from a presumably non-infected herd to an infected one about two months earlier. Three cows died after calving showing signs of haemolytic anaemia. Of the remainder, 38% had PCVs of <25%. However, some of the cattle were born in an endemic area before being moved to the non-infected farm and may not have been naïve at the time of introduction.

Of seven reports of *Theileria* infections in cattle in the U.S.A., most of them in individual animals, two clinical cases were also ascribed to Chitose on its own (one in an 8 year old cow) and Chitose plus variant D (in another old cow).[54, 55]

In project B.AHE.0038, Chitose could not clearly be implicated as a cause of disease on properties in NSW where clinical disease was diagnosed. While present on 40 of 61 properties investigated, it was not the sole variant detected on any of them and was not seen as a pure infection in any of the animals showing severe or moderate anaemia. In contrast, Ikeda was consistently detected on affected properties and was always present in severely anaemic animals, either as a pure or mixed infection (Bailey unpublished 2011). The high frequency of mixed infections of Ikeda and Chitose suggests a common vector on affected properties. That's not surprising and similar to the situations in Japan and Korea.

While the evidence for Chitose being able to cause disease is scanty, we cannot assume that it will be entirely avirulent in high risk classes of animals. This situation is no different to the one we have with the tick fever vaccine so will not necessarily disqualify Chitose from use in a vaccine. Even if a known avirulent strain is used in a live *Theileria* vaccine, the list of "precautions" will be extensive; inclusion of a strain known to be capable of causing disease under certain conditions will merely add a few more precautions to the list.

6 How much is known of the complete genome sequences of Ikeda, Chitose and Buffeli variants?

6.1 Available information

Both Ikeda and Chitose variants have reportedly been sequenced (Sugimoto, unpublished 2011) but nothing has been published on this work.

Because the harmful effects of *T. orientalis* are exerted mainly by the intra-erythrocytic piroplasms, the variable 23kDA and 32-33kDa major surface proteins on the parasites have received most of the attention in phylogenetic studies and in the search for a subunit vaccine. The assumption is that a strong humoral anti-piroplasm response will be adequate to control the harmful effects of the infection.

7 What is the feasibility of developing a recombinant subunit Ikeda vaccine?

7.1 Conclusion

Development of a subunit vaccine that will protect against Ikeda variant of *T. orientalis* is not considered feasible, at least not in the short to medium term irrespective of whether it is delivered as a protein in adjuvant or as a DNA construct. This parasite has a world-wide distribution but a review of "advances and prospects for subunit vaccines against protozoa of veterinary importance" [56], did not even rate it a mention in 2001. Because of this low impact internationally, efforts to develop a recombinant vaccine for *T. orientalis* have been very low key. After some action in the 1990s, little progress has been made in the past decade.

The painfully slow progress being made in the development of vaccines against the other more important vector-borne diseases such as malaria, East Coast fever and bovine babesiosis suggests that funds will be used more productively on efforts directed at other means of control such as chemotherapy and development of a live vaccine.

7.2 Progress in development of a recombinant or synthetic *T. orientalis* vaccine

Our understanding of the immune response in *T. orientalis*-infected cattle is very limited [48] but, despite this, attempts have been made to develop subunit or recombinant vaccines. These attempts focussed mainly on the 32-33kDa (p32-33) major piroplasm surface protein (MPSP) expressed on the surface of the piroplasms[36, 48], the assumption being that a strong anti-piroplasm response is probably all that is required.

Vaccination with non-living components of *T. orientalis* has been attempted by groups in Japan and Korea. In early work, Baek and co-workers in Korea[38, 57, 58] vaccinated calves with crude soluble extracts of parasites and Freunds complete adjuvant followed by a booster dose 4 weeks later with Freunds incomplete adjuvant. When the calves were exposed to tick challenge 9 weeks later, all 20 controls required treatment compared to only 6 of 20 vaccinated calves. Very similar results were obtained when aluminium hydroxide was used as adjuvant and a booster dose given 3-4 months after the first.[59] This effect was thought to be mediated primarily by an immune response directed at the MPSP.[48] Despite these promising results, use of antigen extracted from blood as vaccine posed major difficulties, including the need for sufficient quantities of antigen and the potential for side effects such as neonatal haemolytic anaemia. The group finally developed a recombinant MPSP vaccine.[60] Three vaccinations at 3 week intervals induced an antibody response but did not provide protection against challenge. The authors did not mention how the antigen was expressed or what adjuvant they used.

Onuma and co-workers in Japan[61, 62] produced a recombinant baculovirus expressed MPSP. This expression system produced large amounts of recombinant proteins[63] and, given 4-5 times to splenectomised calves with Freunds complete adjuvant, it reduced the severity of clinical symptoms after challenge and also resulted in lower parasitaemias. They obtained similar results with a synthetic peptide of MPSP.[62, 64] Major obstacles encountered in this work were variant specific protection (Ikeda and Chitose immunity homologous, not heterologous) and the need to use Freunds adjuvant.[64] In follow-up work, the group constructed vaccinia virus recombinants with Chitose and Ikeda MPSP genes respectively [65]. Both recombinants produced type-specific MPSPs that did not cross-react with monoclonal antibodies of the other. However, antisera of immunised mice reacted with both types suggesting the antibodies recognised authentic MPSP molecules. The same group [66] studied cellular immune responses following immunisation of cattle with

recombinant MPSP and, while immunised animals expressed high levels of interferon γ, the authors concluded that immunisation with a cocktail vaccine consisting of different MPSP types may be required under field conditions.[64, 66] However, based on the lack of relevant citations to this promising work, there has been virtually no progress made in the past decade.

More recently, Chinese workers used other expression vectors (pGEM-Easy Vector and pVAX1) to express the p23 gene in combination with p33[67] or on its own.[68] The former study showed enhanced humoral and cellular responses in mice while the latter claimed "better immunogenicity" but did not do any animal work in support of this claim.

Bovine heat shock proteins (HSPs) produced when cells are exposed to stress are very immunogenic and have been studied for their adjuvant effect in *Theileria* vaccine preparation.[69] While peptides and recombinant proteins are easy to manufacture, their immunogenicity is limited mainly because of their non-replicating nature and the lack of activation of antigen-presenting cells. In one study,[69] the genes encoding the MPSP of *Theileria* was expressed as a fusion protein with bovine HSP70 and the adjuvant effect of HSP70 evaluated with regard to antibody response and immunity to challenge. Calves were immunised twice 2 weeks apart with and without Freunds complete or incomplete adjuvants, and challenged after a further 2 weeks. Vaccination extended the prepatent period by 50 days but thereafter the parasitaemias were effectively the same as that of the unvaccinated controls. Addition of Freunds adjuvant did not have a significant effect on the prepatent periods or parasitaemias but the peak parasitaemias in all the groups, including the controls, were very low which made it difficult to come to any real conclusions from this study. HSP-MPSP fusion also enhanced humoral and cellular responses in immunised mice.[69]

Despite the promising results early on, there has been little recent in the development of a recombinant vaccine. To complicate matters further, we know frightfully little of the pathogenesis of the disease (for instance, if it is true that there is poor correlation between parasitaemia and anaemia, [70] which stage in the life cycle should the vaccine target?). We also know very little of the protective immune mechanisms involved (if, as has been reported, infected cattle can develop clinical relapses, [71, 72] a simple humoral response may not be adequate). A lot more needs to be learnt of this parasite before we can target specific antigens and construct a recombinant vaccine.

7.3 Feasibility of developing a recombinant subunit Ikeda vaccine?

Development of recombinant vaccines against the diseases caused by intra-erythrocytic protozoan parasites (malaria, babesiosis and theileriosis) has long been a priority in human and veterinary medicine. However, before anyone considers developing a vaccine against Ikeda variant, we suggest they look at the lack of progress being made, despite concerted efforts, in the development of vaccines against the other more important diseases.

Malaria: It will soon be 30 years since the cloning of malaria parasites with the bold promise that a vaccine would be available in the near future. The 'near future' has long passed and despite millions of dollars being spent in attempts to develop an effective vaccine, malaria still kills more children than any other disease. A candidate *Plasmodium falciparum* vaccine is in Phase III trials but major questions remain over its efficacy and durability. [73]

Largely because of this lack of progress in the search for a recombinant or synthetic malaria vaccine, scientists are now looking closer at the potential development of a live vaccine by using, for instance, irradiated or attenuated organisms and low doses of live sporozoites followed by drug cure.[73] The title of Michael Good's recent paper is: "Our impasse in developing a malaria vaccine". How appropriate!

Bovine babesiosis: A vaccine against *B. bovis* was the focus of a large CSIRO project in the 1980s. Two fusion proteins were identified which, in combination, were almost as protective as the commercially available live vaccine produced at TFC.[74] More work was considered necessary to increase the reliability of antigen expression[75] but the commercial partners withdrew their support and now, almost 20 years later, we are no closer to having this vaccine. A review of progress in the development of a recombinant *Babesia* vaccine by Wendy Brown *et al* in 2006 emphasised how frustrating and difficult the process has been with few promising antigens to consider as vaccine candidates.[76] They further concluded that a protective vaccine, whether delivered as protein in adjuvant or as a DNA construct, will require inclusion of multiple antigens or epitopes of multiple proteins; also that an improved understanding was needed of the mechanisms of protective immunity.

The only option available for the time being is to use live vaccines.[27] These vaccines are based on attenuated organisms derived from culture or infected calves and are available commercially or semi-commercially in a few countries, including Australia, Argentina, Brazil and South Africa.

East Coast fever: Development of a vaccine against ECF was one of the main aims of the International Laboratory for Research on Animal Diseases (ILRAD) when it was established in Kenya in the 1970s. By 2000, an experimental recombinant vaccine based on a sporozoite surface antigen (p67) had been developed that protected 70% of immunised cattle in pen trials[34] and was being evaluated in the field. At the time it was anticipated that the ultimate vaccine will incorporate a mixture of antigens derived from sporozoites and schizonts.[34] However, since then ILRAD changed its focus to become more animal health and productivity oriented and is now the International Livestock Research Institute (ILRI). The ECF vaccine development team was disbanded and the focus in ECF control in Africa has shifted back to the infection and treatment method of the 1970s mentioned in Section 3.2.1.

8 Is Biosecurity Queensland's Tick Fever Centre interested in developing and producing a Theileriosis vaccine as part of it's suite of products?

8.1 Potential development and production of a live vaccine

If the hypothesis that live infections of Buffeli and/or Chitose variants provide adequate protection against Ikeda variant is accepted, the Tick Fever Centre of Biosecurity Queensland will be interested in developing and producing a live vaccine based on one or both of these variants. In most countries, the suggestion of having a live blood-based theileriosis vaccine will be rejected out of hand but, with the precedent of already having a registered, quality controlled live blood-based tick fever vaccine in Australia, there should be no legislative or practical reasons why such a vaccine cannot be developed, registered and produced here.

It is very likely that the procedures and facilities used to produce and evaluate tick fever vaccines can be used to produce a cryopreserved theileriosis vaccine without the need for new or additional resources. The vaccine will probably be issued as a multidose concentrate that is reconstituted and mixed with diluent before use in much the same way as the cryopreserved tick fever vaccine (Combavac). Each batch will be tested for potency before release.

No costings have been done but the cost of production will depend, amongst others on the number of variants to be used in the vaccine. As TFC is a subsidised, Queensland Government-owned service provider, it does not have access to venture capital for new initiatives and funding will be needed for the development, evaluation and registration of a theileriosis vaccine.

9 Bibliography

1. Seddon HR, Albiston HE. Diseases of domestic animals in Australia. Part 4. Protozoan and virus diseases. *Diseases of domestic animals in Australia Part 4 Protozoan and virus diseases*. Canberra: Department of Health, 1966:viii + 257 pp.
2. Stewart NP, Standfast NF, Baldock FC, Reid DJ, de Vos AJ. The distribution and prevalence of Theileria buffeli in cattle in Queensland. *Aust Vet J* 1992;69:59-61.
3. Callow LL. Piroplasms. *Protozoal and rickettsial diseases*. Australian Bureau of Animal Health, Canberra, Australia., 1984:121-182.
4. Izzo MM, Poe I, Horadagoda N, de Vos AJ, House JK. Haemolytic anaemia in cattle in NSW associated with *Theileria* infections. *Aust Vet J* 2010;88:45-51.
5. Bailey G. Benign Theileriosis - an emerging problem? *The Australian Cattle Veterinarian* 2010;54:24-25.
6. Callow LL. *Animal health in Australia. Volume 5. Protozoal and rickettsial diseases.* Australian Government Publishing Service, Canberra, A.C.T. 2601

Australia, 1984.

7. Sugimoto C, Fujisaki K. Non-transforming Theileria parasites of ruminants. In: Dobbelaere AE, McKeever D, editors. *Theileria*. Kluwer Academic Publishers, Boston, 2002:93-106.
8. Gubbels MJ, Hong Y, van der Weide M, et al. Molecular characterisation of the Theileria buffeli/orientalis group. *Int J Parasitol* 2000;30:943-952.
9. Riek RF. Epidemiology and transmission of *Theileria* sp of cattle in Australia. *Aust Vet J* 1982;59.
10. Uilenberg G, Perie NM, Spanjer AAM, Franssen FFJ. Theileria orientalis, a cosmopolitan blood parasite of cattle: Demonstration of the schizont stage. *Res Vet Sci* 1985;38:352-357.
11. Stewart NP. Studies on Theileriosis in Queensland. James Cook University of North Queensland, Townsville, 1990:pp177.
12. Stewart NP, Uilenberg G, De Vos AJ. Review of Australian species of Theileria, with special reference to Theileria buffeli of cattle. *Trop Anim Health Prod* 1996;28:81-90.
13. Roberts FS. A systematic study of the Australian species of the genus *Haemaphysalis* Koch (Acarina : Ixodidae). *Aust J Zool* 1963;11:35-80.
14. Fujisaki K, Kawazu S, Kamio T. The taxonomy of the bovine *Theileria* spp. *Parasitol Today* 1994;10:31-33.
15. Fujisaki K. A review of the taxonomy of Theileria sergenti/buffeli/orientalis group parasites in cattle. *J Protozool Res* 1992;2:87-96.
16. Chae J, Allsopp BA, Waghela SD, et al. A study of the systematics of Theileria spp. based upon small-subunit ribosomal RNA gene sequences. *Parasitol Res* 1999;85:877-883.
17. Jeong W, Yoon SH, An DJ, et al. A molecular phylogeny of the benign Theileria parasites based on major piroplasm surface protein (MPSP) gene sequences. *Parasitology* 2010;137:241-249.
18. Kim JY, Yokoyama N, Kumar S, et al. Molecular epidemiological survey of benign Theileria parasites of cattle in Japan: Detection of a new type of major piroplasm surface protein gene. *J Vet Med Sci* 2004;66:251-256.
19. Yokoyama N, Ueno A, Mizuno D, et al. Genotypic Diversity of Theileria orientalis Detected from Cattle Grazing in Kumamoto and Okinawa Prefectures of Japan. *J Vet Med Sci* 2011;73:305-312.
20. Ota N, Mizuno D, Kuboki N, et al. Epidemiological Survey of Theileria orientalis Infection in Grazing Cattle in the Eastern Part of Hokkaido, Japan. *J Vet Med Sci* 2009;71:937-944.
21. Kamau J, de Vos AJ, Playford M, et al. Emergence of new types of *Theileria orientalis* in Australian cattle and possible cause of theileriosis outbreaks. *Parasites and Vectors* 2011;4:1-10.

22. Terada Y, Ishida M, Yamanaka H. Resistibility to Theileria sergenti infection in Holstein and Japanese black cattle. *J Vet Med Sci* 1995;57:1003-1006.
23. Fukasawa M, Kikuchi T, Shinohara H, Nishida A, Yamagishi T. Genetic parameters for packed red blood cell volume and daily gain during grazing in Japanese Shorthorn calves facing a natural challenge from Japanese theileriosis. *Livest Prod Sci* 2002;77:167-174.
24. Shimizu S, Yagi Y, Nakamura Y, et al. Clinico-hematological observation of calves experimentally infected with Theileria sergenti. *Japanese Journal of Veterinary Science* 1990;52:1337-1339.
25. Hisai T, Oura R, Sekine J. Changes with the season in the number of erythrocytes invaded by Theileria sergenti and packed cell volume of the blood of grazing cattle. *Journal of the Faculty of Agriculture, Tottori University* 1992;28:39-44.
26. Minami T, Ishihara T, Fujita J. Bovine theileriosis and its control in Japan. *Advances in the Control of Theileriosis Proceedings of an International Conference held at the International Laboratory for Research on Animal Diseases in Nairobi, 9-13th February, 1981*, 1981:94-96.
27. Shkap V, De Vos AJ, Zweygarth E, Jongejan F. Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity. *Trends Parasitol* 2007;23:420-426.
28. Ogata T, Tsukamoto D, Sato M, et al. In vitro culture of Theileria orientalis sergenti, and the use of the Bovine-Red Blood Cell-SCID mouse model in-aid of cultivation. *J Protozool Res* 2002;12:49-57.
29. McKeever DJ. Live immunisation against Theileria parva: containing or spreading the disease? *Trends Parasitol* 2007;23:565-568.
30. Katzer F, Ngugi D, Walker AR, McKeever DJ. Genotypic diversity, a survival strategy for the apicomplexan parasite Theileria parva. *Vet Parasitol* 2010;167:236-243.
31. Dolan TT. Dogmas and misunderstandings in East Coast fever. *Trop Med Int Health* 1999;4:A3-A11.
32. McKeever DJ. Bovine immunity - a driver for diversity in Theileria parasites? *Trends Parasitol* 2009;25:269-276.
33. Anon. Galvmed home page and news letters http://www.galvmed.org/about-galvmed. 2011. Retrieved.
34. Morzaria S, Nene V, Bishop R, Musoke A. Vaccines against Theileria parva. In: House JA, Kocan KM, Gibbs EPJ, editors. *Tropical Veterinary Diseases - Control and Prevention in the Context of the New World Order*. New York Acad Sciences, New York, 2000:464-473.
35. Gubbels M-J, Yin H, Bai Q, et al. The phylogenetic position of the *Theileria buffeli* group in relation to other *Theileria* species. *Parasitol Res* 2002;88:S28-S32.
36. Kawazu S. Studies in the classification of the Theileria sergentibuffeli/orientalis/ group of parasites. (Doctoral dissertation). *Bull Natl Inst Anim Health* 1995:77-78.
37. Uilenberg G. Theileria sergenti. *Vet Parasitol* 2011;175:386-386.
38. Baek BK, Kim BS, Choi IH, et al. Immunogenicity and protective efficacy of solubilized merozoite-enriched Theileria sergenti immunogens. II: Protection against natural exposure under field conditions. *Kisaengchunghak Chapchi* 1992;30:201-208.
39. Geysen D. Live immunisation against Theileria parva: spreading the disease? *Trends Parasitol* 2008;24:245-246.
40. Di Giulio G, Lynen G, Morzaria S, Oura C, Bishop R. Live immunization against East Coast fever - current status. *Trends Parasitol* 2009;25:85-92.
41. Stewart NP, De Vos AJ, Shiels I, McGregor W. The experimental transmission of Theileria buffeli of cattle in Australia by Haemaphysalis humerosa. *Aust Vet J* 1987;64:81-83.
42. Stewart NP, de Vos AJ, McGregor W, Shiels I. Haemaphysalis humerosa, not H. longicornis, is the likely vector of Theileria buffeli in Australia. *Aust Vet J* 1987;64:280-282.

43. Stewart NP, de Vos AJ, Shiels IA, Jorgensen WK. Transmission of Theileria buffeli to cattle by Haemaphysalis bancrofti fed on artificially infected mice. *Vet Parasitol* 1989;34:123-127.
44. McFadden AMJ, Rawdon TG, Meyer J, et al. An outbreak of haemolytic anaemia associated with infection of Theileria orientalis in naive cattle. *N Z Vet J* 2011;59:79-85.
45. Heath ACG, Bishop DM, Tenquist JD. Cattle tick Haemaphysalis longicornis in New Zealand - ecology and control. *N Z J Zool* 1980;7:607.
46. Hoogstraal H, al. e. Review of Haemaphysalis (Kaiseriana) longicornis Neumann (resurrected) of Australia, New Zealand, New Caledonia, Fiji, Japan, Korea and northeastern China and USSR, and its parthenogenetic and bisexual populations (Ixodoidea, Ixodidae). *J Parasitol* 1968;54:1197-1213.
47. Chae JS, Levy M, Hunt J, et al. Theileria sp infections associated with bovine fatalities in the United States confirmed by small-subunit rRNA gene analyses of blood and tick samples. *J Clin Microbiol* 1999;37:3037-3040.
48. Boulter N, Hall R. Immunity and vaccine development in the bovine theilerioses. *Adv Parasitol* 2000;44:41-97.
49. Barnett SF, Brocklesby DW. I] Recent investigations into Theileridae of cattle and buffalo in Africa; a mild form of East Coast fever (Theileria parva) with persistence of infection. *Br Vet J* 1966;122:361-370.
50. Zakimi S, Kim JY, Oshiro M, et al. Genetic diversity of benign Theileria parasites of cattle in the Okinawa prefecture. *J Vet Med Sci* 2006;68:1335-1338.
51. Sarataphan N, Kakuda T, Chansiri K, Onuma M. Survey of benign Theileria parasites of cattle and buffaloes in Thailand using allele-specific polymerase chain reaction of major piroplasm surface protein gene. *J Vet Med Sci* 2003;65:133-135.
52. Aktas M, Altay K, Dumanli N. A molecular survey of bovine Theileria parasites among apparently healthy cattle and with a note on the distribution of ticks in eastern Turkey. *Vet Parasitol* 2006;138:179-185.
53. Yu D, Li Y, Chae J, Park J. Genetic diversity in the major surface protein gene of Theileria buffeli in Korean indigenous cattle. *Journal of Veterinary Clinics* 2010;27:501-507.
54. Chae J, Lee J, Kwon O, et al. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene variable (V4) region among and within geographic isolates of Theileria from cattle, elk and white-tailed deer. *Vet Parasitol* 1998;75:41-52.
55. Cossio-Bayugar R, Pillars R, Schlater J, Holman PJ. *Theileria buffeli* infection of a Michigan cow confirmed by small subunit ribosomal RNA gene analysis. *Vet Parasitol* 2002;105:105-110.
56. Jenkins MC. Advances and prospects for subunit vaccines against protozoa of veterinary importance. *Vet Parasitol* 2001;101:291-310.
57. Baek BK, Kim BS, Rhim BM, et al. Immunogenicity and protective efficacy of solubilized merozoite-enriched Theileria sergenti immunogens. III. Characterization of immunodominant peptides. *Korean J Parasitol* 1994;32:111-116.
58. Baek BK, Choi IH, Kim BS, Hansen R, Kakoma I. Immunogenicity and protective efficacy of solubilized merozoite-enriched Theileria sergenti immunogens. I: Protection against homologous stabilate challenge. *Kisaengchunghak Chapchi* 1992;30:133-140.
59. Baek BK, Jung JM, Kim BS. Immunoprophylactic effect of synthetic polypeptide vaccines derived from Theileria sergenti merozoites. *Korean Journal of Veterinary Research* 1996;36:453-461.
60. Park J, Lee S, Chae J, Kwon O, Lee J. Immunization of recombinant membrane protein in Theileria sergenti. *Korean Journal of Veterinary Clinical Medicine* 1999;16:328-331.
61. Onuma M, Kakuda T, Sugimoto C. Theileria parasite infection in East Asia and control of the disease. *Comp Immunol Microbiol Infect Dis* 1998;21:165-177.
62. Onuma M, Kubota S, Kakuda T, et al. Control of Theileria sergenti infection by vaccination. *Trop Anim Health Prod* 1997;29:119S-123S.

63. Kang SW, Kweon CH, Choi EJ, Yoon YD. Expression of major piroplasm protein (p33) of Theileria sergenti (Korean isolate) and its immunogenicity in guinea pigs. *Korean J Parasitol* 1999;37:277-283.
64. Kawazu S, Okumura T, Hirogari Y, et al. A polymorphism observed in the experimentally successful peptide vaccine sequence derived from Theileria sergenti piroplasm major surface antigen (p33). *J Vet Med Sci* 1997;59:829-831.
65. Takasima Y, Xuan X, Matsumoto Y, Onuma M, Otsuka H. Antibodies produced by mice immunized with recombinant vaccinia viruses expressing two different types of a major Theileria sergenti surface antigen (p32) react with the native surface antigen. *Vet Parasitol* 1999;84:65-73.
66. Kakuda T, Sugimoto C, Onuma M. Epitope-mapping of antigen-specific T lymphocyte in cattle immunized with recombinant major piroplasm surface protein of Theileria sergenti. *J Vet Med Sci* 2001;63:895-901.
67. Li J, Xu Y, Zhang X, et al. The p-33-p23 DNA vaccine for Theileria sergenti in cattle. *Chinese Journal of Veterinary Science* 2008;28:1171-1173, 1180.
68. Jin C, Zhang S, Yu L. Cloning and bioinformatics analysis of P23 gene from Theileria sergenti. *Agricultural Science & Technology - Hunan* 2008;9:56-58, 84.
69. Jeong W, Kweon C, Kang S, et al. Adjuvant effect of bovine heat shock protein 70 on piroplasm surface protein, p33, of Theileria sergenti. *Biologicals* 2009;37:282-287.
70. Fukasawa M, Kikuchi T, Konashi S, Nishida A, Yamagishi T. Assessment of criteria for improvement in Theileria orientalis sergenti infection tolerance. *Anim Sci J* 2003;74:67-72.
71. Purnell RE, Rae MC, Deuk SM. Efficacy of imidocarb diproprionate and primaquine phosphate in the prevention of tick-borne disease in imported Hereford heifers in South Korea. *Trop Anim Health Prod* 1981;13:123-127.
72. Kawamoto S, Takahashi K, Onuma M, et al. Rebound phenomenon of parasitemia in splenectomized calves primarily infected with Theileria sergenti. *J Vet Med Sci* 1991;53:127-128.
73. Good MF. Our impasse in developing a malaria vaccine. *Cell Mol Life Sci* 2011;68:1105-1113.
74. Wright IG, Casu R, Commins MA, et al. The development of a recombinant *Babesia* vaccine. *Vet Parasitol* 1992;44:3-13.
75. Mahoney DF. The development of control methods for tick fevers of cattle in Australia. *Aust Vet J* 1994;71:283-289.
76. Brown WC, Norimine J, Goff WL, Suarez CE, McElwain TF. Prospects for recombinant vaccines against Babesia bovis and related parasites. *Parasite Immunol* 2006;28:315-327.