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Assessment of the efficacy of Buparvaquone for the treatment of ‘benign’ bovine theileriosis

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Abstract

There appears to be an emerging disease problem of cattle in eastern states caused by benign *Theileria* infection. Buparvaquone was used in splenectomised calves against two different *Theileria* isolates and proved to be highly effective. The dose confirmation data were provided to the manufacturer in support of its application for a minor use permit, which will give veterinarians access to a specific therapeutic agent where currently one is not available.

Executive summary

Theileria is a common, usually benign, blood parasite of cattle in eastern Australia. However, outbreaks of clinical disease have recently been observed, usually associated with cattle movements to new areas. Mortality rates have been reported to be as high as 30%. This clinical disease has been given the name 'benign' theileriosis to distinguish it from the more pathogenic theilerioses (East Coast fever and tropical theileriosis) overseas. It appears there are three different variants (Ikeda, Chitose and Buffeli) of this 'benign' *Theileria* sp.

There are currently no drugs registered to treat cattle infected with the benign *Theileria* sp., leaving producers and veterinarians with a hopeless situation during these clinical outbreaks. A working group, established to investigate this recent increase in clinical disease, emphasised the need to find a suitable therapeutic agent.

Buparvaquone is registered in about 20 countries for the treatment of East Coast fever and tropical theileriosis. Due to the small potential market in Australia, it is not economically feasible to register the drug here. However, it is worth applying to the Australian Pesticides and Veterinary Medicines Authority (APVMA) for a minor use permit in order to make buparvaquone available in this country.

The objective of this project was to do the Australian "dose confirmation trials" as outlined in the Manual of Requirements and Guidelines of the Australian Pesticides and Veterinary Medicines Authority (APVMA) to obtain such a permit. As part of this, the collection of isolates of the different variants of 'benign' *Theileria* spp was required.

Several isolates of local *Theileria* sp were collected and tested to determine their type. A pure Ikeda variant was collected, as well as a number of mixed-variant isolates. Pure Chitose or Buffeli isolates were not identified.

The efficacy study used splenectomised calves to demonstrate the anti-parasitic activity of the drug. Two groups of calves were inoculated with a mixed-variant isolate and a pure Ikeda isolate. Half of each group was treated with buparvaquone at the dose rate used for the pathogenic theilerioses (2.5mg/kg), and the other half were left as untreated controls.

The treated calves in both groups had dramatic reductions in parasitaemia (89-95% reduction) by day 4 after treatment whereas the parasitaemia in the untreated controls increased over the same period. It was confirmed that buparvaquone was indeed a very effective drug against this parasite.

A minor use permit to enable its use in clinical outbreaks should be pursued as a matter of urgency so that veterinarians dealing with outbreaks will be able to treat cattle with a specific anti-*Theileria* drug.

Once a minor use permit is approved, further work will need to be done to show its effect in clinically affected animals. This will need to be done as field trials during naturally occurring outbreaks since it is difficult to replicate this disease situation due to the usually benign nature of this parasite. Additional work (such as tissue residue depletion studies) may need to be done if buparvaquone is to be fully registered in the future.

Although a relatively minor disease on an industry-wide basis, it is hoped that the availability of an effective drug like buparvaquone will have a very significant benefit for those producers who do face an outbreak of clinical 'benign' theileriosis in the future.

Contents

1 Background

Theileria is a common, usually benign, parasite of red blood cells in cattle in eastern Australia. However, more than 70 widely distributed outbreaks of clinical theileriosis have been reported in eastern and central NSW during the past 4 years. Clinical cases have also been reported on a few Queensland properties. Most of the outbreaks in coastal areas were in cattle introduced from tick-free areas 4 to 6 weeks previously; while the non-coastal outbreaks mostly involved home-bred cattle on properties where coastal cattle had been introduced in recent months. Most outbreaks featured multiple cases and symptoms included anaemia, jaundice, depression and abortion[1]. Mortality rates of up to 30% were reported in some affected mobs.

Workshops on theileriosis were held in Port Macquarie (March 2009) and Sydney (September 2009), and the Animal Health Committee (AHC) subsequently formed a Working Group to further investigate the condition. The Working Group defined the condition seen in Australia as "benign theileriosis" to distinguish it from the highly fatal forms of theileriosis seen overseas (East Coast fever and tropical theileriosis). The exact cause of the condition in Australia is still being investigated, but there appears to be at least 3 variants of *Theileria spp* present in this country (Ikeda, Chitose and Buffeli)[2]. Based on work done in Japan, Ikeda variant appears to be the most likely cause of the disease condition. A MLA-funded project (B.AHE.0038) is also under way to determine the distribution of the different variants in eastern Australia.

There are currently no drugs available for treatment of theileriosis in Australia and the Working Group emphasised the need to find a suitable therapeutic agent. Buparvaquone (BPQ) is registered in about 20 countries for the treatment of East Coast fever and tropical theileriosis and there is evidence in the literature that it is also effective against the agents causing benign theileriosis[3-6]. The drug was originally developed and marketed overseas by Coopers Animal Health (latest parent company Intervet-Schering Plough). Because of the small potential market locally, the company does not consider it feasible to register BPQ in Australia; it is willing however to apply to the Australian Pesticides and Veterinary Medicines Authority (APVMA) for a Minor Use Permit in order to make BPQ available in this country. The sole objective of this project is to do the local "dose confirmation trials" as outlined in the Manual of Requirements and Guidelines of the APVMA.

2 Project objectives

i) Obtain isolates containing the required *Theileria* variants from field cases for use in the treatment trials
ii) Confirm the dose efficacy of BPQ in controlled trials against Australian variants of *Theileria spp.*

3 Methodology

3.1 Isolation of *Theileria* variants

The objective was to capture two to three different isolates against which the efficacy of buparvaquone could be evaluated. The initial intention was to find a pure isolate of each of the Buffeli, Ikeda and Chitose variants.

Blood was either collected from naturally infected calves which developed significant parasitaemias after splenectomy, or blood was collected from field cases and passaged through splenectomised calves to amplify the parasites. Calves used were of similar origin to those used in the dose efficacy trials (see 3.2.1 below). Parasitised blood collected from splenectomised calves was collected during a rising parasitaemia to produce stabilates for future work. Stabilates were made by mixing blood with an equal volume of cryoprotectant (20% polyvinyl pyrrolidone (PVP)). These stabilates were frozen and stored in liquid nitrogen.

Blood samples from each stabilate donor calf were also collected and sent to Elizabeth Macarthur Agricultural Institute (EMAI, NSW I&I)) to be tested by a *Theileria* variant typing polymerase chain reaction (PCR) test. This was done to determine which variants were present in each stabilate. PCR testing revealed that the majority of isolates captured contained a mixture of variants. It is worth noting though that the PCR test was a newly established and non-validated test and did not always show up a positive result, even though parasites could be seen in blood smears. This may implicate a problem with the DNA extraction technique or the sensitivity of the PCR. Adjustments were made to the testing procedure over time which seemed to improve the consistency of results.

A pure Ikeda isolate was identified and the stabilate designated J36. This isolate came from a property near Camden in NSW which had previously experienced an outbreak of clinical 'benign' theileriosis during which the organism was demonstrated to be pure Ikeda by PCR. Additional blood samples were collected from remaining animals some months after the outbreak and inoculated into splenectomised calves to amplify the parasite, from which the stabilates were made. The stabilate donor calves were also tested by PCR and shown to be positive for the Ikeda variant only.

Pure Buffeli and Chitose isolates could not be identified and it was decided to test the efficacy of buparvaquone against the pure Ikeda isolate and a mixed variant isolate. A stabilate designated as J26 was made from a naturally infected calf born at the TFC Wacol. The J26 donor calf tested positive to the 3 different variants by PCR, but there was insufficient of this stabilate for the trial so it was inoculated into another calf for amplification. This led to the production of stabilate J33 which tested positive for Buffeli and Chitose only in the PCR. It is not known whether the Ikeda variant was left behind during the stabilate amplification or whether it came through at a low level and was not detected by the PCR.

3.2 Buparvaquone efficacy

3.2.1 Animals used

Calves derived from the Tick Fever Centre's (TFC) Specific Pathogen Free (SPF) breeding herd located at Dalby were used for this project. Their dams were Charolais/Brahman cross heifers, mated to Shorthorn bulls.

Calves were transported to a calf shed at TFC, Wacol, as a batch when 3-5 weeks of age, where they were reared on milk replacer for 6 weeks and introduced to a steam-flaked barley ration plus oaten chaff. Once weaned from milk, they remained on this ration until disposal. Soon after arrival at TFC, each calf was splenectomised under general anaesthesia to increase the animals' susceptibility to haemoparasites (as well as to detect relapses to naturally transmitted contaminant haemoparasites, such as *Theileria* spp). By removing a major component of the calves' defence against haemoparasitic infection, a more reliable parasitaemia resulted; and it was therefore possible to demonstrate the antiparasitic effect of the drug without the calves' own immune system (which involves the spleen) reducing the parasitaemia and thus confounding the result.

Calves were then selected from the calf batch for use in this project, whilst the remaining calves were used for TFC's routine vaccine production. Calves were selected for phenotypic and size uniformity.

The calves used were those surplus to vaccine production requirements which meant the entire project could not be performed all at once. Instead, the project was divided into two trials: the first trial to assess the efficacy against a mixed *Theileria* infection; and the second trial to assess the efficacy against the Ikeda variant of *Theileria.*

Additional calves of the same type were also used to amplify *Theileria* isolates.

All calf usage was subject to animal ethics approval (approval number SA 2010/05/315).

3.2.2 Housing

Calves were housed in concrete-floored pens. Pens were hosed out once daily. Calves were fed a steam flaked barley ration with oaten chaff.

3.2.3 Inoculation of calves

The first group of 10 calves were inoculated intravenously via the jugular vein with 2.5ml of thawed stabilate J33 whose donor had tested positive by PCR for both Buffeli and Chitose variants.

For the second trial, 12 calves were inoculated intravenously with 2.5ml of thawed J36 stabilate (containing the Ikeda variant) with the intention that the first 10 calves to develop patent parasitaemias could be used. This was done because of the variable prepatent periods during the first trial.

3.2.4 Monitoring

Following inoculation, the *Theileria* parasitaemias of calves were monitored by stained thin tail tip blood smears. Smears were collected twice weekly from the second week after inoculation. Smears were fixed with 100% methanol and stained for 10-15 minutes with 10% Giemsa strain. Stained smears were examined under oil immersion light microscopy at 1250x magnification. Approximately 20 high-power fields were examined on each smear and the average number of parasitised blood cells per field recorded. Where the parasitaemia was less than 1 per field, the following numerical parasitaemias were assigned for calculations:

Just less than 1 per field:	0.8	(ie 4 parasitised cells in 5 fields)
Less than category above but more than 1 in 20 fields:	0.2	(ie 1 parasitised cell in 5 fields)
1 in approximately 20 fields:	0.05	

As the parasitaemia increased, blood was also collected into EDTA-treated capillary tubes for packed cell volume (PCV) measurement to ensure calves did not develop life-threatening anaemia.

Following allocation to treatment and control groups, calves were monitored by thin blood smears on days 3, 4 and 6 or 7 after treatment and/or allocation.

A 5ml blood sample was also collected into EDTA at the time of treatment or allocation to the control group. These blood samples were sent to EMAI to be tested by a *Theileria*-typing PCR to determine which *Theileria* variant(s) was/were present at the time of treatment.

3.2.5 Allocation of calves to treatment groups

Calves were allocated to treatment or control groups by stratified randomisation by stratifying on parasitaemia so that each group would be composed of calves with a similar array of parasitaemias.

3.2.6 Therapeutic agent and Treatment

The drug used was Butalex® which contains 50mg/ml buparvaquone. This drug was kindly imported and donated by Intervet Schering Plough. Calves were treated with a single

intramuscular injection at a dose rate of 2.5mg/kg (1ml per 20kg) as recommended by the manufacturer for use against the other more pathogenic *Theileria* species. The weight of each calf was estimated using a weight band/tape as scales were not accessible within this quarantine zone at TFC facilities. We have found these weight bands to be reasonably close to the actual weight in the past. The weight of each calf was in the vicinity of 190kg (range 155-210kg) in trial 1 and 155kg (range 145-165kg) in trial 2.

The intention was that calves would be treated when the parasitaemia reached 10-30 parasitised erythrocytes per high-power field examined on blood smears. However, due to delays in the buparvaquone arriving from overseas, treatment was delayed in the first trial and so the parasitaemia of some calves was much higher (as much as 80 per field).

3.2.7 Evaluation of an effect

A therapeutic effect was determined in two different ways. Firstly, by calculating the percentage change in parasitaemia of each calf between day 0 (day of treatment) and day 4 after treatment. This was done for both treatment and control groups, where the day 0 in the control group was the day they were allocated to the control group. These percent changes in parasitaemia were used to calculate the statistical significance of the results. Statistical significance was determined using a two sample t-test with unequal variance in Microsoft Excel.

The other method to calculate the therapeutic effect was to calculate the average parasitaemia in each group on day 4 after treatment and calculate a percentage difference between the treatment and control groups, similar to the method Marley et al [7] used. This gave a single value for the effect, rather than comparing two values (one for the treatment group and one for the control group) as in the first method, but did not allow a statistical significance to be calculated.

Day 4 after treatment was chosen to evaluate efficacy based on previous experience with the use of buparvaquone and other anti-Theileria drugs at TFC. This allowed time for the red blood cells containing degenerating parasites to be removed from circulation.

4 Results and discussion

4.1 Isolation of *Theileria* variants

A pure isolate of the Ikeda variant was collected and cryopreserved as a stabilate for future use. However, a pure Buffeli and a pure Chitose isolate were not identified. Most isolates collected and tested contained mixtures of two or all three variants. Blood samples collected from calves which had been inoculated with cryopreserved blood that was shown to contain all three variants, only tested positive to Buffeli and Chitose. When cryopreserved blood from this calf was inoculated into additional calves (for trial 1), most of these recipient calves only tested positive for the Buffeli variant, with only two showing a weak positive result for the Chitose variant. It is not known whether these results are due to a loss of a variant with each subsequent inoculation (syringe passage) or due to diagnostic sensitivity issues with the PCR. However, determining this lied outside the scope of this project.

4.2 Buparvaquone efficacy

A single treatment with buparvaquone caused degeneration of parasites and a decrease in the parasitaemia. Table 1 shows the results of the first trial against the Buffeli variant, and table 2 shows the results of the second trial involving the Ikeda variant.

The calves in trial 1 were inoculated with stabilate J33 which was made from blood containing Buffeli and Chitose variants, as determined by PCR testing. However, PCR testing of blood samples taken from the J33-inoculated calves at the time of allocation to each group showed that

in 7 of the 9 calves, only the Buffeli type was present; Chitose was only detected in two of the calves, and then only with a weak positive result.

Table 1. Buparvaquone efficacy against the 'Buffeli' variant of *Theileria* spp.

	Parasitaemia (per HPF*)		% Change in
Calf	**Day 0**	**Day 4**	**parasitaemia**
Treated			
9702	45	2	-96%
9704	36	0	-100%
9709	9	0.2	-98%
9713	80	40	-50%
9717	55	0	-100%
Treated mean	**45.0**	**8.4**	**-89%**
		(95% confidence limits)	**(-116%, -62%)**
Control			
9714	2	8	300%
9719	25	44	76%
9721	75	150	100%
9726	80	155	94%
Control mean	**45.5**	**89.3**	**142%**
		(95% confidence limits)	**(-25%, 310%)**
		t-test P value	**0.020**

* HPF = High Power Field (1000x magnification)

The parasitaemias of some calves reached quite high levels in trial 1 due to a delay in the new batch of Butalex arriving from Germany. This batch failed to arrive in time and so calves were treated with the existing batch on hand, which was 1 month beyond the expiry date at the time of treatment. Had the new batch arrived when expected, the calves in trial 1 would have been treated at lower parasitaemias (for example, 10-30) as was originally planned.

Table 2. Buparvaquone efficacy against the 'Ikeda' variant of *Theileria* spp.

	Parasitaemia (per HPF*)		% Change in
Calf	**Day 0**	**Day 4**	**parasitaemia**
Treated			
9736	14	0.2	-99%
9739	12	0.2	-98%
9748	14	0	-100%
9749	22	0	-100%
9753	18	4	-78%
Treated mean	**16.0**	**0.9**	**-95%**
		(95% confidence limits)	**(-107%, -83%)**
Control			
9730	18	30	67%
9733	9	10	11%
9741	10	24	140%
9750	20	33	65%
9754	15	25	67%
Control mean	**14.4**	**24.4**	**70%**
		(95% confidence limits)	**(13%, 127%)**
		t-test P value	**0.001**

* HPF = High Power Field (1000x magnification)

There was a considerable change in the parasitaemia following treatment in both trials. In trial 1, there was an 89% reduction in parasitaemia by day 4 after treatment, compared with untreated controls whose parasitaemia continued to rise (by 142% on average). This difference in the percentage change in parasitaemia was statistically significant (P=0.02). Likewise, in trial 2, there was a 95% reduction in parasitaemia following treatment whereas controls continued to rise by 70%. Again, this difference was highly statistically significant (P=0.001).

It should be noted though that, although parasites were still seen in the blood smears of some treated calves four days after treatment, these parasites were very degenerated based on morphological characteristics. A vital stain to distinguish viable from non-viable parasites was not available, and therefore any parasites seen were counted. However, it is extremely unlikely that these parasites would have been viable. In fact, the calf in trial 1 that had a parasitaemia of 40 parasitised cells per high power field on day 4 after treatment, only had 2 parasitised cells on day 6 after treatment and no detectable organisms by day 7. All treated animals had no detectable parasites (degenerated or otherwise) in blood smears by day 6-7 after treatment, meaning efficacy was 100% by day 6-7.

Therefore, since degenerating, and probably non-viable, parasites are included in the counts, the estimates of drug efficacy at day 4 are more than likely an underestimate of the true efficacy.

The alternative method of calculating an estimate of drug efficacy by comparing the average parasitaemia on day 4 after treatment of treated calves with that of untreated controls returns the following estimates:

Trial 1: Buffeli variant	91%
Trial 2: Ikeda variant	96%

These drug efficacies are very good, but statistical significance cannot be calculated with this method. Again, they are most likely underestimates since degenerating parasites are included in the calculation.

Whilst a single treatment with buparvaquone caused a rapid reduction in parasitaemia and all calves were blood smear negative by day 7 after treatment, the treatment did not cause sterilisation of the infection. All 5 treated calves in trial 1 relapsed 2-3 weeks after treatment, whereas in trial 2, only 2 calves relapsed within this time frame. Monitoring was not continued beyond this to see whether the other calves would eventually relapse. However, sterilisation of infection was not the objective. In the clinical setting, the aim of the treatment will merely be to rapidly kill the parasites to allow the animal time to mount an immune response to keep the infection under control.

5 Success in achieving objectives

It proved difficult to obtain pure isolates of each *Theileria* variant in the short time between the start of the project and the need to start the efficacy study. Although it is not difficult to find *Theileria* infected animals given the endemic nature of this organism, most infections appear to consist of a mixture of variants. It was also problematic that the PCR was still under development and not yet optimised. Many negative results were obtained despite organisms being present in high enough numbers to be detected under the microscope, especially early on in the project, but this improved and results were reasonably consistent later on in the project.

Buparvaquone was shown to be highly effective at rapidly reducing the parasitaemia of both *Theileria* isolates tested. These results are similar to those reported for this drug against *Theileria* spp in Japan as well as against East Coast fever and tropical theileriosis[3-6]. Hence this work confirms that the dose used against these other *Theileria spp* overseas is also effective against

the different variants of our 'benign' *Theileria* species. This data can be used by Intervet-Schering Plough in their application to the APVMA for a minor use permit to allow buparvaquone (and Butalex® in particular) to be used in cattle which are clinically affected with 'benign' theileriosis.

We gratefully acknowledge the support (including importation and provision of Butalex® for the trial) of Intervet-Schering Plough in pursuing this research to have a drug available to treat 'benign' theileriosis despite the very small return on investment.

We would also like to acknowledge Graeme Eamens and Jocelyn Gonsalves from EMAI (NSW I&I) for their PCR work to type the isolates collected.

6 Impact on meat and livestock industry – Now and in five years time

Buparvaquone has been shown to be effective in rapidly killing *Theileria* organisms. This will provide efficacy data to support an application for a minor use permit to allow clinically affected cattle to be treated where currently there is no effective treatment available.

Should the permit be approved and should the drug prove to be effective in the clinical setting, then this will be an excellent outcome for producers, particularly those that introduce stock to the *Theileria* endemic areas.

Being a relatively minor disease problem when viewed on an industry-wide basis, it is anticipated that there will be a very small market for Butalex® which means full registration would not be a viable proposition for Intervet-Schering Plough. However, enough Butalex® will need to be used to justify Intervet-Schering Plough continuing to import and hold stock of it.

7 Conclusions and recommendations

Buparvaquone is highly effective at rapidly reducing the parasitaemia of Buffeli as well as the Ikeda *Theileria* isolates and a minor use permit should be sought to allow cattle clinically affected with 'benign' theileriosis to be treated.

It is recommended that the supply of buparvaquone for treatment of clinical 'benign' theileriosis be restricted to veterinary professionals as for Schedule 4 products, since it will not be fully registered and to prevent widespread and indiscriminate use of the product.

It is also necessary that further field work be performed to confirm buparvaquone's effectiveness in clinical outbreaks once a minor use permit is approved. It must be noted that this current work was done in splenectomised calves to more effectively show the anti-parasitic effect of the drug. Further work is needed to evaluate the efficacy in animals experiencing clinical 'benign' theileriosis. Because infection does not always lead to a clinical disease this work will need to be done in outbreak situations and will require a coordinated approach between private and government veterinarians so that outbreaks can be monitored to generate data to determine its effectiveness.

Promotion of this work should be done to ensure veterinarians are, firstly, aware that there is a drug that is effective against *Theileria* parasites and that it is available under minor use permit (if approved). Veterinarians should also be made aware that there is currently no data to show that it is effective in the clinical setting, but that further work is needed to generate this data and that their cooperation will be required in reporting outbreaks so that trials can be conducted during these outbreaks. Secondly, further promotion should be done to veterinarians once efficacy in clinical outbreaks has been determined.

It is also recommended that other research into 'benign' theileriosis continue or begin such as determining the geographical distribution of the different *Theileria* variants; determining the vectors, and the distribution of these vectors, for the different variants; and looking at ways clinical cases can be prevented rather than treated.

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