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Merozoites of Theileria Orientalis Buffeli Reduce Parasitosis Following Challenge by Ticks Infested with T.Orientalis Ikeda

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Abstract

Background: An investigation aimed to confirm whether immunisation with the “benign” buffeli genotype of *Theileria orientalis* could reduce the parasitosis of the virulent ikeda genotype.

Methods: Calves were inoculated intravenously or subcutaneously with bovine blood containing merozoites of *T.orientalis* buffeli. When recipients became positive, they and control animals were challenged with unfed nymphs of *Haemaphysalis longicornis* ticks infested as larvae with *T.orientalis* ikeda.

Results: All calves became positive for the challenge within 12 days after tick application. In the immunised calves, the first wave of parasitosis with *T.orientalis ikeda* from 4-6 weeks was reduced significantly by >80% before the infestation declined into the carrier state by 9 weeks.

Conclusions: The results confirm the field experience with theileriosis in endemic zones where the carrier state appears to prevent clinical disease despite repeated, seasonal tick infestations with virulent genotypes of the parasite. This method offers a means to reduce the severity of the first wave of theilerial parasitosis after tick challenge and recover associated production losses.

1. Background

The intracellular protozoal parasite *Theileria orientalis* has rapidly spread across South-eastern Australia, substantially impacting local cattle industries since 2006. Given that *Theileria buffeli*, causing “benign theileriosis” had been present in Queensland since 1912 (Stewart et al.,1992; 1996), the new syndrome was termed “**B**ovine **A**naemia caused by the T *heileria* o *rientalis* **g**roup” (BATOG; Eamens et al., 2013a). Molecular techniques have since enabled discrimination of the *T.orientalis* genotypes and identified genotypes “ikeda” and “chitose” as those causing clinical disease (Kamau et al., 2011; Eamens et al., 2013a,b; Bogema et al., 2015). The basis for the current classification uses the major piroplasm surface protein (MPSP), which is expressed in the intraerythrocytic stage of *T. orientalis* and conserved to some extent among different geographic isolates. It has been widely used for molecular epidemiological studies of *T. orientalis* in Japan (Ota et al., Sivakumar et al., 2012), Korea (Park et al., 2017), Kenya, and Australia (Izzo et al., 2010; Kamau et al., 2011; Bogema et al., 2015). Currently, 11 genotypes of *T. orientalis* (type 1 or chitose, type 2 or ikeda, type 3 or buffeli, types 4–8, and N1-N3) had been identified based on MPSP gene sequences (Sivakumar et al., 2014; Bogema et al., 2015). Of these genotypes, 1 and 2 cause the majority of clinical disease in cattle, with the phylogenetic cluster chitose A mostly associated with the ikeda genotype in clinical cases (Jenkins et al., 2015). On farms impacted by the parasite, NSW-DPI have estimated an average cost of $59K for dairy producers and $11.6K for beef producers, which equates to AUD $131/head for dairy cattle and AUD $67/head for beef cattle (Bailey 2012). Similar losses have been reported from Victoria (Perrera et al., 2014). The total cost of theilerosis in Australia was estimated at around $20 m pa nationally (Lane et al., 2015). Recovered cattle remain carriers of theilerial genotypes (Eamens et al., 2013b; Hammer et al., 2016) and several studies have

indicated that the carrier state in dairy cattle did not compromise subsequent productivity (Perrera et al., 2014; Lawrence et al., 2019).

Interestingly, inoculation of naïve cattle with blood stages of *T.orientalis* genotypes does not cause clinical disease (Hammer et al., 2016; Gibson, 2017), but a carrier state is established and parasitosis is detectable by PCR in blood for greater than 30 months (Hammer et al., 2016; unpublished). Irrespective of the theilerial genotype(s) present, the carrier state arising after natural tick-borne infection appears to prevent recurrence of clinical disease following seasonal tick challenge in endemic regions (Izzo etal, 2012; Kamau et al., 2011; Eamens et al., 2013b). This has been witnessed by the progression of the epidemic curve through eastern and southern Australia and New Zealand, with mainly newborn calves and introduced cattle remaining susceptible to clinical disease in endemic zones (Bailey, 2012; Jenkins et al., 2015). Similarly, the widespread presence of *T.orientalis* buffeli carrier cattle in Queensland has been attributed to the low prevalence of virulent genotypes and/or clinical theileriosis in that state (de Vos, 2011). In endemic regions, infestations of *T.orientalis* buffeli in susceptible cattle were clearly outpaced by virulent theilerial genotypes ikeda and chitose in both Gloucester and Dorrigo (Jenkins et al., 2015; DE, unpublished), negating any opportunity to induce protection. Leaving vector competence aside, some type and level of immunity exists in carrier cattle which resembles “premunity” (Neal et al., 1969), interfering with the severity of subsequent challenge infections. This is witnessed in the long histories of early “vaccinations” against leishmania, malaria, East Coast fever, babesiosis and poultry coccidiosis (“precocious strains”) (see McAllister, 2014).

Consistent with the circumstantial and historical evidence for the operation of “premunity” against repeated seasonal tick infestation with *T.orientalis*, this study examined whether immunisation with blood stages (merozoites) of *T. orientalis* buffeli could reduce the severity of subsequent *T.orientalis* ikeda infestation. This study also enabled an opportunity to determine whether *H. longicornis* nymphal ticks infected as larvae on an infected calf could transmit that infection as nymphs, to an uninfected second host, and then retain that ability to re-infect a third host as adult ticks.

2. Materials And Methods

2.1 Theilerial stabilates.

T.orientalis ikeda. Blood was collected from the jugular vein of a donor cow into lithium heparin blood collection tubes at Camden NSW, 28 ml of which was subsequently inoculated into a splenectomised calf at the Tick Fever Centre, Wacol, Qld. Approximately 1 month after inoculation, a stabilate (designated J36) was produced using 100 ml of blood collected into sodium heparin mixed with an equal volume of 20% polyvinylpyrrolidone (PVP 40,000; pH 7.2) cryoprotectant solution. This blood mixture was decanted into each of 5 ml cryotubes The cryotubes were then placed in the vapour phase of liquid nitrogen for 15 minutes before the stabilate was lowered into the liquid nitrogen for storage. The stabilate contained 2×10^8 merozoites per ml. For infection of ticks, unfed adult *H. longicornis* ticks which had previously fed on a splenectomised calf that had been inoculated with a thawed 5 ml cryotube of J36 stabilate, were

placed on a subsequent splenectomised calf (3604) and transmitted *T. orientalis* ikeda. Additional clean larval ticks were then fed on this *T. orientalis* Ikeda-infested calf to themselves become infected. The engorged larval ticks were then collected and allowed to moult to nymphs in the incubator before allpication.

T.orientalis buffeli. A blood stabilate (J46) was similarly prepared from a cow at Bairnsdale, Victoria and stored in liquid nitrogen at the TFC. To prepare the blood inoculum for this trial, splenectomised calf 3584 was inoculated with a thawed 5 ml cryotube of stabilate intravenously and blood collected some months later.

2.2 Calves and infection.

The trials in this study conformed with requirements for animal health and well-being under the University of Sydney Animal Ethics Committee (AEC) permit 2018/1328.

Fifteen Holstein (neutered male) calves aged 3–4 months were purchased from Leppington Pastoral Company (LPC, Cobbity, NSW) and housed on pasture at The University of Sydney's Pye Farm, Greendale NSW. Calves were weighed and bled to confirm their negative theilerial status by AusDiagnostics® Multiplex-Tandem PCR (MT-PCR) before being randomly assigned to 3 treatment groups, each of 5 animals. Group 1 calves were premedicated with 100 mg of the antihistamine chlorpheniramine maleate (Histamil ®) and 15 mg dexamethasone (Dexapent®) by intramuscular injection, to lower the risk of any reaction from blood incompatibility. After 30 min, each calf was given a 5 ml intravenous (IV) infusion of fresh infected blood obtained from an infected steer at the TTFC, (steer 3584, infected with *T.orientalis* buffeli blood stabilate). Previous screening by PCR indicated a specific infection containing 1.12×10^5 gene copies of *T.orientalis* buffeli per ul blood. The inoculum contained 1.3×10^6 merozoites of *T.orientalis* buffeli per ml. All calves became PCR positive within 28d. Group 2 calves were to be infested with 100 *H.bancrofti* nymphs (infected with *T.orientalis* buffeli as larvae) from TFC under patches, but these ticks died on steer 3584. Consequently, *H.longicornis* larvae were fed on steer 3584, moulted, and approx. 200 unfed nymphs were applied to each calf in Group 2 under backline calico patches as described by Marendy et al. (2019). Ticks were collected and removed after 6 days when they were engorged. When these 5 calves failed to become PCR positive after 5 weeks, 5 ml of fresh infected blood containing 9×10^8 *T.orientalis* buffeli merozoites per mL (from steer 3584) was inoculated subcutaneously (SC) into each calf. All these 5 calves were PCR positive for *T.orientalis* buffeli within 21d.

Thirteen weeks after Group 1 calves and 28 days after Group 2 calves were inoculated with *T.orientalis* buffeli blood, approximately 200 unfed *H.longicornis* nymphs, previously infected with *T.orientalis* ikeda as larvae at TFC, were placed under backline patches on each of the 15 calves (including the uninoculated group 3 controls). The ticks were collected from each calf after 6 days, counted and pooled, before posting to the Biosecurity Sciences Laboratory (BSL, Brisbane, Qld), to moult to adults; 10 were also placed into 100% ethanol for PCR. When moulted and their exoskeletons had hardened (Marendy et al.,2019), around 50 of these unfed adult *H.longicornis* were applied to each of 3 naïve calves to

determine if the original *T.orientalis* ikeda infection persisted through the nymphal stage on the uninfected calves and survived through the moult to adult ticks.

All calves were monitored visually daily, with 5 ml blood collected weekly into EDTA vacuum tubes (Vacuette, Griener Bio-one) from day 14 post-inoculation of buffeli blood and from days 14–62 after *T. orientalis* ikeda infected, tick challenge.

2.2 Sample analyses.

PCV was measured by centrifuging blood in micro-haematocrit tubes (Beckman Coulter centrifuge). Diagnostic PCR was performed by DNA extraction from 200 ul blood samples using the MagMax™ CORE Nucleic Acid Purification kit (ThermoFisher Scientific Inc.). A KingFisher Duo™ Prime Magnetic Particle Processor completed DNA isolation.

Theilerial PCR runs were conducted with 11 eluted DNA samples, one indicator sample, MasterMixes (Theileria), oil, water, and step 2 strips loaded separately into the AusDiagnostics® Easy-Plex™ Processor, which provided sequences for theilerial genotypes ikeda, buffeli, chitose, and type 5. Both automatic assay set-up and Step 1 PCR were accomplished in this machine. Samples were then transferred into the Bio-rad CFX96 analyser to perform the Step 2 PCR. Real-time PCR Ct-values were collected for data analysis. Relative gene copy numbers per ul of blood (GP/ul) were calculated for both pan-theileria and theilerial genotypes from spiked standards in the assay.

2.3 Statistical analysis.

Statistical analysis was performed using GenStat ™. Raw data were $\log_e$ transformed and two linear mixed models for PCV and ikeda gene copies per µL were developed for each study as appropriate. The fixed effects were Day, Treatment and the interaction between Day and Treatment. The random effect was Animal ID. P-values were calculated for each fixed effect in each model to determine significance. For significant fixed effects, the differences in the predicted means for each factor level were compared to the Least Significant Differences (LSDs) at significance level 0.05 to determine whether pairwise comparisons were significant. If the interaction fixed effect was significant, no further pairwise comparisons were determined for the other fixed effects.

Predicted means for PCV and GC/ul for theilerial genotypes within treatment groups were presented with standard error (SE) bars.

3. Results

3.1 Immunisation with *T.orientalis* buffeli.

Both intravenous (IV) and subcutaneous (SC) inoculation of bovine blood infected with *T.orientalis* buffeli produced parasitosis detectable by PCR within 4 weeks of inoculation. Over the 13 weeks before challenge, the parasitosis in Group 1 (IV) reached a mean peak of 5,063 GC/ul of *T.orientalis* buffeli by 5

weeks, decreasing and stabilising 9–13 weeks after initial inoculation between means of 1292 and 876 GC/ul (Table 1). By comparison, at the time of challenge, 4 weeks after inoculation SC, Group 2 calves had a mean parasitosis of 1547 GC/ul (Table 1). Following the *T. orientalis* Ikeda infected, tick challenge, the parasitosis with *T.orientalis* buffeli remained relatively steady in both Groups 1 and 2 over the next 60 days at < 2500 GC/ul.

Table 1
Parasitosis of *T.orientalis* buffeli from calves in Groups 1 & 2 after inoculation of infested blood.

Group treatment	Days after tick infestation								
	-62*	-56	-28	0	12	18	35	47	62
IV (Gp1)	9144 +/- 85	5063 +/- 72	1292 +/- 21	876 +/- 0.67	133 +/- 0.65	3 +/- 0.2	3 +/- 0.2	3 +/- 0.3	2 +/- 0.2
SC (Gp2)	na	na	na	1547 +/-0.7	157 +/- 0.65	20 +/- 0.65	24 +/- 0.6	20 +/- 0.4	12 +/- 0.4
Results are expressed as mean GC/ul blood +/- SE.									
* 4 weeks after inoculation of blood IV; na = not applicable									

Following the initial inoculations of blood into Group 1, it was revealed that 2 calves in the control Group 3 exhibited pre-existing low parasitoses with *T.orientalis* ikeda of < 500 GC/ul, presumably from infections acquired *in utero* or postnatally. This meant that these 2 calves were excluded from the control group (3 calves) for statistical analysis, but were included for analysis as a separate group (of 2 calves) after tick infestation.

3.2 Effects of *T.orientalis* buffeli on *T.orientalis* ikeda after tick challenge.

All 15 calves in the 3 groups became positive for *T.orientali*s ikeda within 12 days after infestation (DAI) from the application of the 200 infected *H.longicornis* nymphs. The parasitosis in the control groups followed a typical pattern in peaking around 5 weeks (39 DAI) after infestation at a mean 69734 GC/ul before declining to < 2000 GC/ul blood by 62 DAI (Fig. 1, Table 2). The parasitosis in the 3 treatment groups were significantly reduced between 30 and 85% on sampled days during the first wave of parasitaemia from 25–39 DAI (Fig. 1, Table 2). In parallel, the PCV in the control group decreased by 16–20% after infestation to a mean of 25% by 39 DAI, significantly reduced compared to the SC immunised group 2 (Table 3) and remained significantly lower than group 2 up to 62 DAI (Table 3).The PCV of the IV-immunised group 4 was only significantly higher than control group 1 on 32DAI (Table 3).

Table 2
Parasitosis of *T.orientalis* ikeda in treatment groups after challenge with infested *H.longicornis*.

Groups	Day 12	Day 18	Day 25	Day 32	Day 39	Day 47	Day 62
Control Gp3 (3)	189 (97)^{ab}	9180 (4690)^{a}	33134 (18585)^{a}	30303 (15500)^{a}	68734 (8969)^{a}	18251 (9150)^{a}	84 (588)^{a}
IV (5)	94 (37)^{a}	2800 (1110^{a}	1124 (445)^{c}	3446 (1360)^{b}	9740 (6414) ^{b}	10027 (6253) ^{a}	2855 (1588) ^{b}
*Controls + ve (2)	1181 (835)^{b}	9063 (6409) ^{a}	6741 (4767) ^{ab}	11464 (8107)^{ab}	6815 (4374) ^{b}	810 (632) ^{b}	422 (228) ^{a}
SC (5)	488 (193)^{b}	9200 (3640)^{a}	4020 (1590)^{b}	3648 (1440)^{b}	9154 (9180) ^{b}	3637 (4224) ^{ab}	432 (331) ^{a}
Results are expressed as mean GC/ul blood +/- SE; * the 2 calves in Gp3 animals found positive for *T.orientalis* prior to tick challenge							
Within columns, data with different superscripts are significantly different ($p < 0.05$).							

Table 3
Changes in haematocrit in treatment groups after challenge with infested *H.longicornis*.

Groups	Day 12	Day 18	Day 25	Day 32	Day 39	Day 47	Day 62
Control (3)	32 ^{a}	38.3 ^{a}	31.7 ^{a}	25 ^{a}	26.7 ^{a}	27 ^{a}	27.3 ^{a}
IV (5)	27.8 ^{a}	30.8 ^{a}	32 ^{a}	29.8 ^{b}	30.2 ^{ab}	30.4 ^{ab}	28.2 ^{ab}
*Controls + ve (2)	35 ^{a}	33.5 ^{a}	36 ^{a}	27.5 ^{ab}	29.5 ^{ab}	32 ^{b}	31.5 ^{bc}
SC (5)	28.8	33.6 ^{a}	31.6 ^{a}	28.6 ^{ab}	31.6 ^{b}	33.2 ^{b}	31.8 ^{c}
Results are expressed as % PCV.							
Within columns, data with different superscripts are significantly different ($p < 0.05$).							

3.3 Persistence of theilerial infestation between larval and adult stages of *H.longicornis*..

Moulted adult ticks derived from the application of *T.orientalis* ikeda- infested nymphs of *H.longicornis* onto 15 uninfected calves (as a second host) were reapplied to 3 naive calves as third hosts. Blood

samples at 18 DAI were positive for *T.orientalis* ikeda with 920, 3150 and 10,280 GC/ul in the 3 calves, indicating that the ticks retained infectivity through 2 moults.

4. Discussion

Both intravenous (IV) and subcutaneous (SC) inoculation of bovine blood infected with *T.orientalis* buffeli produced parasitosis detectable by PCR within 4 weeks, consistent with previous reports (Hammer et al., 2016; Gibson, 2017). In each case the parasitosis appeared to peak around 5–8 weeks before stabilising at around 2000–10000 GC/ul. It was also noted when *H.bancrofti* ticks failed to adequately feed and died, that *H.longicornis* nymphs failed to transmit *T.orientalis* buffeli to infect naive calves. These results were consistent with previous vector studies indicating that *H.bancrofti* and *H.humerosa* were likely vectors for *T.orientalis* buffeli in Queensland (Stewart et al., 1987a,b). *H. longicornis* occurs in the coastal areas of Victoria and New South Wales and extends northwards as far as Gympie in Queensland but is absent from large areas of Northern Australia where *Theileria* sp (*T.orientalis* buffeli) is present (Reik, 1982).

Prior inoculation of *T.orientalis* buffeli-infected blood containing between 6.5×10^6 GC/μL (iv) or 4×10^9 GC/μL (sc) and allowed to "consolidate/incubate" for 13 and 4 weeks, respectively, before *T.orientalis* ikeda- infected ticks were applied, significantly reduced the initial parasitosis of *T.orientalis* ikeda over the next 50 days. It was also observed that 2 calves already serendipitously parasitised with < 500 GC/ul *T.orientalis* ikeda on arrival at 4 months of age, presumably *in utero* or post-natally, were also significantly "protected" from the first wave of parasitosis following tick challenge. These outcomes consolidated several older theilerial reports from Japan and Korea (Baek et al., 1982; Minami et al., 1981; Onuma et al., 1997) with *T.orientalis* sergenti, which has been confirmed as *T.orientalis* (Stewart et al., 1996). A cryopreserved vaccine containing 2×10^8 red blood cells containing *T.orientalis* [sergenti] per dose "had an inhibitory effect on the clinical manifestation of theileriosis" with a need for proliferation of the inoculum (Ishihara, 1962) but this was not developed further. Production of an attenuated whole blood vaccine against *T. orientalis* [sergenti] occurred in Korea but outcomes were not reported and challenge appeared to use blood stabilate (Baek et al.,1992). Later, sonicated *T. orientalis* [sergenti] merozoites produced significant reductions in parasitosis after 3 months, among recipients receiving 2 doses of 100 mg in complete Freund's adjuvant subcutaneously, 1 month apart, and subjected to field infestation from 2–5 months after initial vaccination (Baek et al., 1992). Unfortunately, the trial was terminated after 5 months as all controls and 20% (4/20) of vaccinates required treatment with diminazene (Berenil®) for anaemia (Baek et al., 1992). A recombinant MPSP vaccine for *T.orientalis* [sergenti] utilised 3 vaccinations at 3 week intervals, producing an antibody response but no protection against challenge (Park et al, 1999).

Overall, these reports and the current trial support the premise that prior infestation with either *T.orientalis* ikeda or buffeli merozoites, effectively generates a state of "premunity" which mitigates the severity of a subsequent tick challenge with *T.orientalis* ikeda (Neal et al., 1969; Stewart et al., 1992). This situation reflects field experience where recovered cattle may harbour multiple theilerial genotypes in the carrier

state. It should be noted that inoculation of merozoites of *T.orientalis* ikeda into susceptible cattle does not appear to cause the clinical episodes of theileriosis that follows tick infestation (Hammer et al., 2016; this study). Such a method of “immunisation” may be a feasible means of control against ticks carrying the virulent theilerial genotypes.

These results are also consistent with the susceptibility of introduced cattle and calves in endemic regions with multiple theilerial genotypes. As noted with the kinetics and age-related infections of calves and introduced stock (Jenkins et al., 2015; Swilks et al., 2017) and in naïve animals at Dorrigo, *T.orientalis* buffeli infestations are the slowest to develop (Emery et al., unpublished). Results indicated that infections with the virulent genotypes (ikeda and chitose) occurred earlier with maximal parasitosis around 5–6 weeks before declining, whereas parasitosis with *T. orientalis* buffeli was detectable in an increasing proportion of naïve animals up to 3–4 months before stabilising (Jenkins et al., 2015; Emery et al., unpublished). All animals remained carriers of all four genotypes in the AusDiagnostics® PCR kit for at least 6 months and > 80% of ticks sampled from Dorrigo and Stroud contained DNA from the four theilerial genotypes (ikeda, chitose, Type 5 and buffeli). The earlier appearance of the virulent genotypes negates any opportunity for endemic *T.orientalis* buffeli to generate any host-protective capability before challenge with virulent genotypes.

This study is the first confirmation of speculations that the carrier state persisting in cattle which have recovered from the initial parasitosis with virulent theilerial genotypes, establishes some type of “protection” to subsequent, seasonal tick challenge. As recommended by de Vos (2011), dose-response studies with single or mixed genotypes are required to establish vaccination potential. Field trials in endemic regions with high levels of tick infestation harbouring multiple theilerial genotypes are also vital to determine the robustness of the method, and whether there is any further “protection” conferred to parasitosis induced by subsequent tick challenges. Therefore, this outcome is being repeated in a field trial providing an intense natural challenge and to test homologous protection using *T.orientalis* ikeda blood. It may be that prior immunisation with merozoites from any genotype(s) of *T.orientalis* may reduce the severity of parasitosis following challenge with virulent genotypes as appears to be the case in numerous regions of infested with *T.orientalis*.

In an additional observation on the vector competence of *H.longicornis* for *T.orientalis* ikeda, unfed nymphs infected as larvae effectively transmitted the parasite to naïve second host calves and after collection and moulting to adult stages, could readily transmit *T.orientalis* ikeda to a further group of naïve calves. This indicated that the tick retained an initial infection acquired in the larval stage, through two moults to the adult stage and did not require any “boosting” from the second host. In the field, this implies that the nymphal stage could engorge on companion or wildlife second hosts and still remain infective for cattle as their third host.

5. Conclusions

Calves inoculated SC or IV with blood infested with merozoites of *T.orientalis* buffeli became positive for the parasite by PCR around 28 days after infestation (DAI) and did not develop clinical signs over the following 9 weeks. Following the application of 200 *H.longicornis* nymphs harbouring *T.orientalis* ikeda, the first peak of parasitosis occurring from 25–47 DAI was reduced by around 80% in immunised calves compared to control animals. These results are consistent with the lack of clinical theileriosis in carrier cattle in endemic regions and strongly suggest that host regulation maintains theilerial populations of the virulent genotypes below levels causing clinical disease, despite repeated seasonal tick challenges. The results also imply that prior vaccination with blood forms of the parasite could reduce deaths and production losses incurred from the first wave of parasitosis following tick infestation with virulent theilerial genotypes ikeda and chitose A.

Declarations

Competing interests. The authors declare that they have no competing interests

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Figures

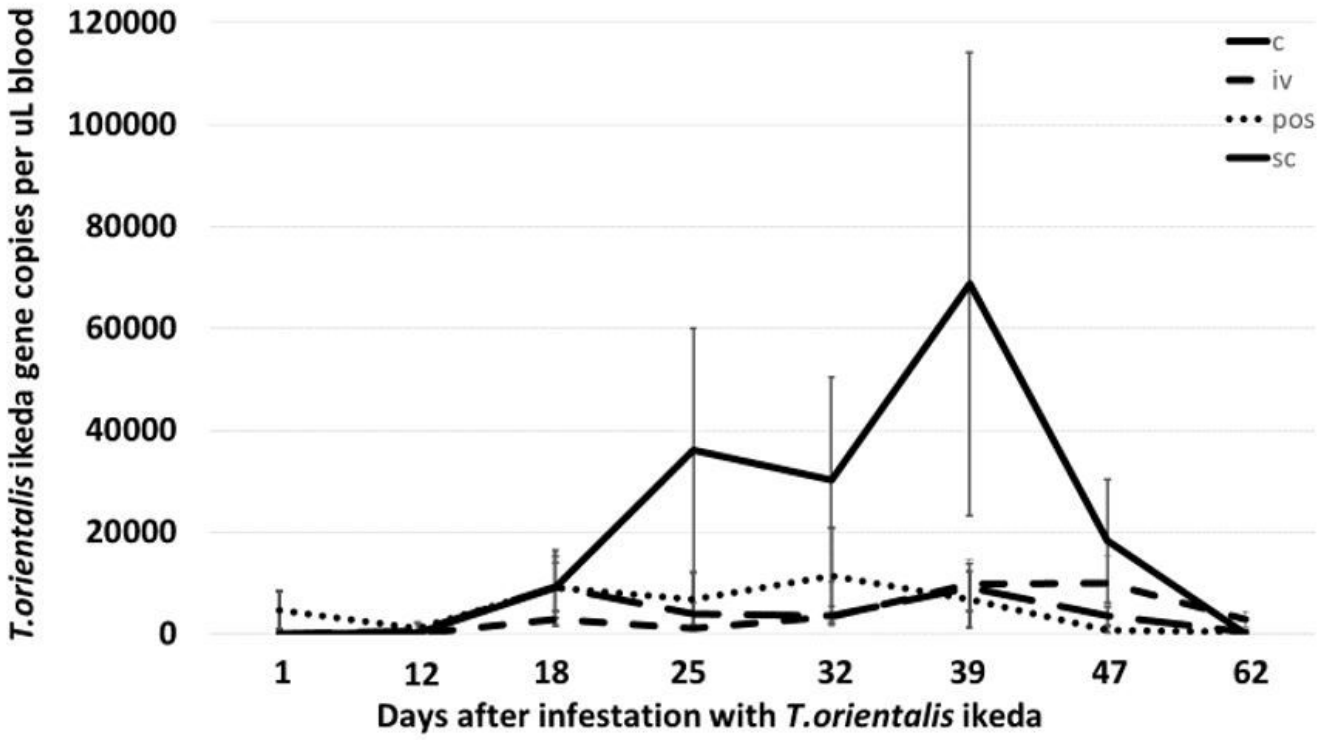

Figure 1

Group mean parasitoses for T.orientalis ikeda (GC/ per ul blood +/- SD) following infestation with 200 H.longicornis nymphs (see M&M). Group comparisons include: uninfected controls (Gp 3; 3 calves, solid line); previously infected controls (2 calves, dotted line); groups of 5 calves immunised with T.orientalis buffeli either IV (Gp1; short dash) or SC (Gp2; long dash).

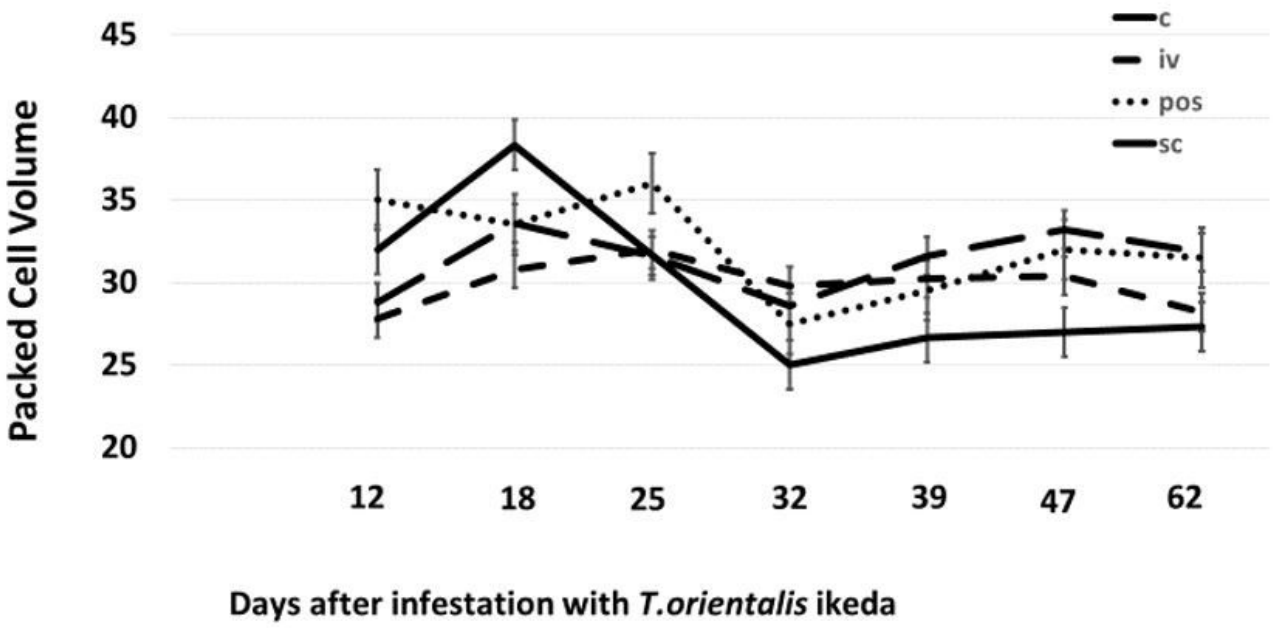

Figure 2

Group mean haematocrit (% +/- SD) following infestation with 200 H.longicornis nymphs (see M&M). Group comparisons include: uninfected controls, (Gp 3; 3 calves, solid line); previously infected controls (2 calves, dotted line); groups of 5 calves immunised with T.orientalis buffeli either IV (Gp1; short dashes) or SC (Gp2; long dash).