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Research paper

Merozoites of *Theileria orientalis* buffeli reduce the parasitaemia of *T. orientalis* ikeda following tick challenge

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ABSTRACT

Theileria orientalis is a tick-borne protozoal parasite causing anaemia and death in susceptible cattle. This investigation aimed to confirm whether immunisation with the "benign" buffeli genotype of *T. orientalis* could reduce the parasitaemia of the virulent ikeda genotype. Calves were inoculated intravenously or subcutaneously with bovine blood containing merozoites of *T. orientalis* buffeli and when recipients became positive by PCR, they and control calves were challenged with unfed nymphs of *Haemaphysalis longicornis* ticks infected as larvae with *T. orientalis* ikeda. All calves became positive for the challenge within 12 days after tick application. In the immunised calves, the first wave of parasitaemia with *T. orientalis ikeda* from 4 to 6 weeks was reduced significantly by >80 % before the parasite burden declined into the carrier state by 9 weeks. The parasitaemias in two calves which exhibited low infections with *T. orientalis* ikeda shortly after arrival, were also significantly reduced after tick challenge. The results confirm the previous studies on immunity to *T. sergenti* in Japan, and 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 parasitaemia after tick challenge and possibly recover associated production losses.

1. Introduction

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,2013b; 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., 2009; Sivakumar et al., 2012), Korea (Park et al., 2017), Kenya, New Zealand and Australia (Izzo et al., 2010; Kamau et al., 2011; Sugimoto and Fujisaki, 2002; 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, New South Wales Department of Primary Industry (NSW-DPI) have estimated an average cost of AUD59,000 for dairy producers and AUD11,600 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 (Perera et al., 2014). The total cost of theilerosis in Australia was estimated at around AUD20 million 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

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subsequent productivity (Perera 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; Lawrence et al., 2018), but a carrier state is established and parasite DNA is detectable by PCR in blood for more 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 (Emery et al., 2021). 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; Emery et al., 2021), 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 leishmaniosis, malaria, East Coast fever, babesiosis and poultry coccidiosis ("precocious strains") (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 parasitaemia and associated disease. This study also enabled an opportunity to determine whether *Haemaphysalis longicornis* nymphal ticks infected as larvae 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. A clinical case of bovine theileriosis at Camden NSW was confirmed as *T. orientalis* ikeda at the Elisabeth Macarthur Agricultural Institute (EMAI) by genotypic PCR (Bogema et al., 2015). Blood was collected from the jugular vein into lithium heparin blood collection tubes (Beckton-Dickenson, Sydney, Australia), 28 mL of which was subsequently inoculated into a splenectomised calf at the Tick Fever Centre (TFC), 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 to produce the stabilate consisting of 40 aliquoted doses. The cryotubes were then placed in the vapour phase of liquid nitrogen for 15 min 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 fed previously as nymphs 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-infected calf to ingest the parasite. The engorged larval ticks were then collected and allowed to moult to nymphs in the incubator as described by Marendy et al. (2019) before application to the experimental calves.

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 (IV) and blood collected 2–3 months later when the parasitaemia exceeded 1×10^6 merozoites per ml blood.

2.2. Calves and infection

Fifteen Holstein (neutered male) calves aged 3–4 months were purchased from Leppington Pastoral Company (LPC, Cobbity, NSW) and kept 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 infusion IV of fresh infected blood obtained from an infected steer at the TFC, (steer 3584, infected with *T. orientalis* buffeli blood stabilate). To confirm infection, screening of this steer by PCR indicated a specific infection containing 1.12×10^4 gene copies of *T. orientalis* buffeli per µL blood. Consequently, the donor was bled 2 weeks later and the inoculum contained 1.3×10^6 merozoites of *T. orientalis* buffeli per ml when examined on a blood smear. All inoculated calves became PCR positive within 28 days. 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 around 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 21 days after inoculation, and did not exhibit any clinical signs of theileriosis over the ensuing 10 weeks. Following the initial inoculations of blood into Group 1, it was found that 2 calves in the control Group 3 exhibited pre-existing low parasitaemias with *T. orientalis* ikeda of <500 gene copy numbers per µL blood (GC/µL), presumably from infections acquired *in utero* or postnatally (but not from ticks as calves were reared indoors). 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 (Group 4) after tick infestation.

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 and Group 4). The ticks were collected and counted from each calf after 6 days. Those ticks collected for the 13 calves in Groups 1–3 were pooled and posted to the Biosecurity Sciences Laboratory (BSL, Brisbane, Qld), to moult to adults; a randomly selected 10 ticks 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. Blood from these recipient calves were examined by PCR for both ikeda and buffeli genotypes of *T. orientalis*.

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 14 to 62 days after infection (DAI) with ticks transmitting *T. orientalis* ikeda.

2.3. Sample analyses

The Packed Cell Volume (PCV) was measured by centrifuging blood in micro-haematocrit tubes (Beckman Coulter centrifuge). Diagnostic PCR was performed by DNA extraction from 200 μL blood samples using the MagMax™ CORE Nucleic Acid Purification kit (ThermoFisher Scientific Inc.). A KingFisher Duo™ Prime Magnetic Particle Processor completed DNA isolation. Ticks were harvested and placed in 100 % ethanol for 3 days before being cut longitudinally. The half with mouthparts was kept while DNA was extracted from the other segment for analysis (Emery et al., 2021).

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 a CFX96 Touch Real-Time PCR Detection System (Biorad) to analyse the Step 2 PCR. Real-time PCR Ct-values were collected for data analysis. Relative gene copy numbers per μL of blood (GC/ μL) were calculated for both pan-theileria and theilerial genotypes from spiked standards in the assay. Samples were designated as "strongly positive" where cycle take-off (Ct) values were <15; "positive" with Ct values 15–30; and, "negative" with Ct values >30.

2.4. Statistical analysis

Statistical analysis was performed using GenStat ™. Raw data were $\log_e$ transformed and two linear mixed models for PCV and ikeda GC/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/ μL for theilerial genotypes within treatment groups were presented with standard deviations (SDs).

3. Results

3.1. Immunisation with T. orientalis buffeli

Both IV and SC inoculation of bovine blood infected with *T. orientalis* buffeli produced parasitaemia detectable by PCR within 4 weeks of inoculation. Over the 13 weeks before challenge, the parasite burden in Group 1 (IV) reached a mean peak of 5063 GC/μL of *T. orientalis* buffeli by 5 weeks, decreasing and stabilising 9–13 weeks after initial inoculation between means of 1292 and 876 GC/μL (Table 1). By comparison, at the time of challenge, 4 weeks after inoculation SC, Group 2 calves had a mean parasitaemia of 1547 GC/μL (Table 1). Following the *T. orientalis* ikeda infected, tick challenge, the parasitaemia with *T. orientalis* buffeli remained relatively steady in both Groups 1 and 2 over the next 60 days at <2500 GC/μL (Table 1).

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

All 13 calves in the 3 main groups 1–3 became positive for *T. orientalis* ikeda within 12 DAI from the application of the 200 infected *H. longicornis* nymphs. The parasitaemia in the 3 control calves in Group 3 followed a typical pattern in peaking around 5 weeks (39 DAI) after infestation at a mean 69,734 GC/μL before declining to <2000 GC/μL blood by 62 DAI (Fig. 1, Table 2). The parasite burden in the 3 treatment groups (Groups, 1, 2 & 4) were significantly ($p = 0.006$) reduced between 30 and 85 % on sampled days during the first wave of parasitaemia from 25 to 39 DAI (Fig. 1, Table 2). The PCV at the time of tick application was similar for each of groups 1–4 at around 30 (Table 3). The PCV in the 3 control calves in Group 3 decreased by 16–20 % after infestation to a mean of 25 % by 39 DAI, significantly ($p < 0.05$) reduced compared to the SC immunised Group 2 (Table 3) and remained significantly ($p < 0.05$) lower than Group 2 up to 62 DAI (Table 3).The PCV of the 2 animals in group 4 was only significantly ($p = 0.04$) higher than control group 3 on 32 DAI (Table 3). There were no significant differences ($p > 0.05$) in the weight gains of the treatment groups over the 2 months after tick challenge.

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 13 uninfected calves in Groups 1–3 (as a second host) were reapplied to 3 naïve calves as third hosts. Blood samples at 18 DAI were positive for *T. orientalis* ikeda with 920, 3150 and 10,280 GC/μL in the 3 calves. At 25 DAI, the parasitaemia had increased to 5,210, 39,332 and 81,220 GC/μL, respectively in the 3 calves, indicating that the ticks retained infectivity through 2 moults. *T. orientalis* buffeli DNA was not detected in any calf at 18 or 25 DAI. Due to unseasonal bushfires, the study was terminated at this point. The 10 engorged nymphal ticks which had been placed into ethanol also were negative in PCR for *T. orientalis* ikeda DNA (Ct >30) ; 6 were positive for *T. orientalis* buffeli DNA, reflecting the blood meal ingested from the second host.

4. Discussion

Both IV and SC inoculation of bovine blood infected with *T. orientalis* buffeli produced parasitaemias detectable by PCR within 4 weeks, consistent with previous reports (Hammer et al., 2016; Lawrence et al., 2018). In each case the parasite burden appeared to peak around 5–8 weeks before stabilising at around 2000–10000 GC/μL. 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 naïve calves. These results were consistent with previous vector studies

Table 1
Parasitosis of *T. orientalis* buffeli from calves in Groups 1 & 2 after inoculation of blood containing merozoites of the parasite.

Group treatment	Days after tick infestation								
	−62*	−56	−28	0	12 DAI	18 DAI	35 DAI	47 DAI	62 DAI
Group1 *T. buffeli* IV	9144 +/- 85	5063 +/- 72	1292 +/- 21	876 +/- 0.67	133 +/- 0.65	3 +/- 0.2	3 +/- 0.2	3 +/- 0.3	2 +/- 0.2
Group 2 *T. buffeli* SC	na	na	na	1547 +/-0.7	157 +/- 0.65	20 +/- 0.65	24 +/- 0.6	20 +/- 0.4	12 +/- 0.4

Results at days after tick infestation (DAI) are expressed as mean gene copies (GC)/μL blood +/- SE. Tick infestation occurred on day 0 with 200 *H. longicornis* nymphs infected with *T.orientalis* ikeda.

* 4 weeks after inoculation of blood IV; na = not applicable.

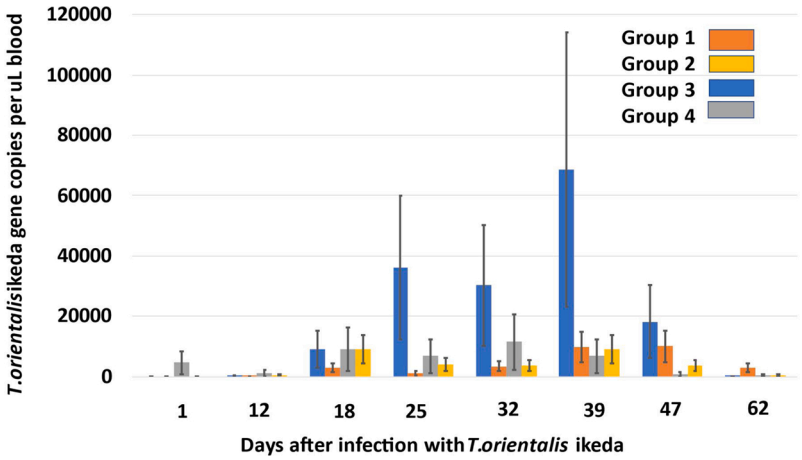

Fig. 1. Group mean parasitaemias for *T.orientalis* ikeda (GC/μL blood +/- SD) following infection with 200 *H.longicornis* nymphs. For each DAI, data from left to right are: Group 3 (controls); Group 1 (IV); Group 4 (2 carriers); and, Group 2 (SC).

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

Group (n)	12 DAI	18 DAI	25 DAI	32 DAI	39 DAI	47 DAI	62 DAI
Group 1; *T. buffeli* IV (5)	94 +/-37^{a}	2800 +/-1110^{a}	1124 +/- 445^{c}	3446+/- 1360^{b}	9740+/- 6414 ^{b}	10,027+/- 6253 ^{a}	2855+/- 158) ^{b}
Group 2: *T. buffeli* SC (5)	488 +/- 193^{b}	9200 +/- 3640^{a}	4020 +/- 1590^{b}	3648 +/- 1440)^{b}	9154+/-9180 ^{b}	3637 +/- 4224 ^{ab}	432 +/- 331 ^{a}
Group 3; controls (3)	189 +/- 97^{ab}	9180 +/- 4690^{a}	33,134 +/- 18,585^{a}	30,303 +/- 15,500^{a}	68,734 +/- 8969^{a}	18,251 +/- 9150^{a}	84 +/- 588^{a}
Group 4 (2)*	1181 +/- 835^{b}	9063 +/- 6409 ^{a}	6741 +/- 4767 ^{ab}	11,464 +/- 8107^{ab}	6815 +/- 4374)^{b}	810 +/- 632 ^{b}	422+/- 228 ^{a}

Results at days after infestation (DAI) are expressed as mean GC/μL blood +/- SE.
*The 2 calves (Gp4) 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 PCV in treatment groups after challenge with infested *H. longicornis*.

Group (n)	Day 0	Day 12	Day 18	Day 25	Day 32	Day 39	Day 47	Day 62
Group 1; IV (5)	29.7^{a}	27.8 ^{a}	30.8 ^{a}	32 ^{a}	29.8 ^{b}	30.2 ^{ab}	30.4 ^{ab}	28.2 ^{ab}
Group 2; SC (5)	29.2^{a}	28.8 ^{a}	33.6 ^{a}	31.6 ^{a}	28.6 ^{ab}	31.6 ^{b}	33.2 ^{b}	31.8 ^{c}
Group 3;Control (3)	30.3^{a}	32 ^{a}	34.3 ^{a}	31.7 ^{a}	25 ^{a}	26.7 ^{a}	27 ^{a}	27.3 ^{a}
Group 4, (2)	32.5^{a}	35 ^{a}	33.5 ^{a}	36 ^{a}	27.5 ^{ab}	29.5 ^{ab}	32 ^{b}	31.5 ^{bc}

Results are expressed as % PCV.
Within columns, data with different superscripts are significantly different ($p<0.05$).

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 (Riek, 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 parasitaemia of *T. orientalis* ikeda over the next 50 days. It was also observed that 2 calves found parasitised with <500 GC/μL *T. orientalis* ikeda shortly after arrival at 4 months of age and presumably infested *in utero* or post-natally, were also significantly "protected" from the first peak of parasitaemia following tick challenge. These outcomes consolidated several previous theilerial reports from Japan and Korea (Baek et al., 1982; Minami et al., 1981 unpublished; Onuma et al., 1997) with *T. sergenti*, which has been confirmed as *T. orientalis* (Stewart et al., 1996). In those reports, 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 a whole blood vaccine against *T. orientalis* [sergenti] occurred in Korea but outcomes were not published and challenge appeared to use blood stabilate (cited in Baek et al., 1992). Onuma et al. (1997) also reported that inocula of blood containing live *T. sergenti* gave variable levels of protection against tick challenge in Japan, an effect attributed to genetic variations in the MPSP p32 protein across the country. Whole blood vaccines were not pursued due to the possible transfer of viruses (Baek, et al., 1992). Later, sonicated *T. orientalis* [sergenti] merozoites produced significant reductions in parasite burden 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 to 5 months after initial vaccination (Baek et al., 1992). The trial was terminated after 5 months as all controls and 20 % (4/20) of vaccinates required treatment with diminazene (Berenil®) for theileria (Baek et al., 1992). In each of these early trials, the genotypes of *T. orientalis* were not known. 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). However, in a limited study, Onuma et al. (1997) immunised 5 calves with 4–5 doses of recombinant "I" (ikeda) and "C" (chitose) MPSP in Freund's adjuvant or liposomes and reported qualitative "vaccine effects" after challenge with a stabilate containing both "variants"(genotypes). This study was the first to indicate that cross-protective immunity could be generated against

genotypes of *T. orientalis*, but the levels of parasitaemia were not reported.

Overall, these earlier reports and the current trial support the premise that prior infection with blood containing 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 (Kubota et al., 1996; Eamens et al., 2013a; Emery et al., 2021). It should be noted that inoculation of merozoites of *T. orientalis* ikeda or buffeli into susceptible cattle does not appear to cause the clinical episodes of theileriosis that follow tick infestation (Hammer et al., 2016; this study). This outcome may result from the lack of a substantial proliferation of lymphocytes containing the macroschizont stage of *T. orientalis* as occurs with *T. parva*, where such blood inocula may cause severe disease (Emery et al., 1982). However, this method of "immunisation" may be a feasible means of control against ticks carrying the virulent genotypes of *T. orientalis*. The mechanism by which any "protection" is mediated has not been investigated, but is likely to reflect similar responses to those which regulate theilerial populations in carrier cattle.

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., 2021). Results consistently indicate that infections with the virulent genotypes (ikeda and chitose) occurred earlier with maximal parasite burdens around 5–6 weeks before declining (Kubota et al., 1996), 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, 2020; Emery et al., 2021). In these investigations, the cattle tested 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 the peak parasitaemia with the virulent genotypes.

This study is the first confirmation of observations in Australia that the carrier state persisting in cattle which have recovered from the initial parasitaemia with virulent theilerial genotypes in regions with endemic theileriosis, establishes some type of "protection" to subsequent, seasonal tick challenge(s) (J. Hammer & C. Shirley, unpublished). Previous studies in Japan and Korea have reported similar results for *T. sergenti* (*T. orientalis*; Ishihara, 1962; Baek et al., 1992). 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 pertinent to determine the robustness of the method, and whether there is any further "protection" conferred to infections 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 blood merozoites from any genotype(s) of *T. orientalis* may reduce the severity of the parasite burden following challenge with virulent genotypes as appears to occur in many regions with endemic *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 third-host calves without reinfection during the nymphal feed. The untimely termination of the study at 25 DAI prevented a complete analysis of the first wave of parasitism, but the prepatent period and kinetics of the parasitaemia appeared similar to the first transmission as nymphs to naive second hosts. It was noted that a sample of several engorged nymphs were deemed "negative" in PCR for *T. orientalis* ikeda DNA with a Ct value >30, confirming that they did not ingest any ikeda parasites in the blood meal on the second host. This result also confirms that when moulted and re-applied as unfed adults, further feeding by the adult stage is needed to mature and inoculate sporozoites (Kimbita and Silayo, 1997; Marendy et al., 2019). The results indicated that the ticks 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. While microbial pathogens are retained in salivary glands during successive tick moults (Piesman and Schneider, 2002), the situation for protozoal parasites with 3-host tick vectors has not been examined to our knowledge. It was also interesting that *T. orientalis* buffeli was not detected in the 3 calves infested with the adult ticks; this likely reflects the incompetence of *H. longicornis* as a vector for *T. orientalis* buffeli in Australia (Stewart et al., 1987b; this study) or the early termination of this component of the trial. Our ongoing research is examining the host-parasite relationship to determine how this is achieved, whether this effect extends to other theilerial genotypes and if there are detrimental effects on tick survival.

5. Conclusions

Calves inoculated SC or IV with blood containing merozoites of *T. orientalis* buffeli became positive for the parasite by PCR around 28 DAI and did not develop clinical signs over the following 9 weeks. Following the application of 200 *H. longicornis* nymphs carrying *T. orientalis* ikeda, the first peak of parasitosis occurring from 25 to 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.

CRediT authorship contribution statement

David Emery gained funding and compiled the paper, oversaw field trials. **Susan de Burgh** & **Therese Hoang Hieu Hanh Dinh** conducted the experiments and data analysis. **Peter Rolls** and **Phillip Carte** arranged blood inocula, tick numbers, infected and moulted ticks.

Animal ethics

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.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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