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Transmission of Theileria orientalis in cattle

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Abstract

Theileria are blood-borne intracellular protozoal parasites belonging to the phylum Apicomplexa. Previously considered a benign parasite in Australia, outbreaks of clinical disease resulting from *Theileria orientalis* genotypes have been reported in Australia since 2006. Since this time, outbreaks have become widespread in south-eastern Australia, resulting in significant adverse impacts on local dairy and beef industries. This project was the first investigation into the possible biological and mechanical vectors involved in the rapid spread of the parasite. Findings implicate the bush tick, *Haemaphysalis longicornis,* as the most likely biological vector, and identifies by PCR, Theilerial DNA in pools of mosquitoes from endemic areas and lice from infected calves. Further work on active transfer from ticks was confounded by lack of suitable vectors and histopathology for sporozoites in the salivary glands from engorged, PCR-positive ticks was equivocal. Biting arthropods could be involved in mechanical (horizontal) transmission as inoculation of just 100µl of infected blood resulted in successful infection of recipient calves. Although mechanical transfer of infected blood did not result in clinical Theileriosis, calves remained positive for 15 months, with obvious implications for husbandry procedures and disease epidemiology. Transplacental and colostral transfer were also investigated, but were not demonstrated in a limited initial study.

Executive summary

Previously considered a benign parasite in Australia, outbreaks of clinical disease resulting from *Theileria orientalis* genotypes have been reported in Australia since 2006. Since this time, outbreaks have become widespread in south-eastern Australia, resulting in significant adverse impacts on local dairy and beef industries. The disease is estimated to cost the Australian cattle industry around AUD $20m annually. Clinical signs of *T. orientalis* are mostly associated with the sequellae from anaemia and involve the pathogenic genotypes, Ikeda and Chitose. These clinical signs can include depression, weakness, anorexia, pale mucous membranes, lymph node swelling, tachypnoea, tachycardia, dyspnoea, pneumonia, jaundice, late-term abortions, dystocia, still births, pyrexia, metritis, and mortality. The vectors, transmission and the early stages of the disease development are not known, but from other models of vector-borne tick fevers, are presumed to involve ticks and early development in the local lymph node, before fever occurs coincident with detection of piroplasmic stages in erythrocytes around 7-9 days after infestation.

To assist with the development of control measures as well as to define the features and transmission of *T.orientalis* towards vaccination and chemotherapy, this project involved:

- identification of the vector(s) for transmission of *T.orientalis;*
- establishing possible conditions for passive transfer of infection;
- establish the seasonality of transmission related to clinical disease; and,
- classification of haematophagous arthropods in Victoria.

Vector(s) for transmission of *T.orientalis.* A wide range of blood-sucking arthropods were collected from areas and herds which had experienced clinical cases of theileriosis. A range of ticks from cattle, companion animals and wildlife, mosquitoes and biting flies were collected by hand or from traps before being tested by PCR for *T.orientalis*. Lice were collected from mechanically infected calves (below). Biting flies returned negative results, but one of ten pools of 100 mosquitoes was positive for *T.orientalis* DNA and two of the remaining nine pools were just below the threshold. In contrast, *T.orientalis* DNA was readily detected in the tick, *Haemophysalis longicornis,* from herds with recent outbreaks of clinical theileriosis. Ticks from wildlife were negative. The study also identified the range of arthropods collected, confirming that the range of *H.longicornis* coincided with the current incidence of clinical Theileriosis. With recent outbreaks of the disease in New Zealand, this adds to the body of international evidence for the role of *H.longicornis* as the definitive host in the life cycle and transmission of the parasite. A seasonal scarcity of the tick in 2014 precluded studies to confirm active infection using salivary glands or saliva from *H.longicornis.* Subsequent attempts to demonstrate sporozoites of *T.orientalis* by histopathology of salivary glands from PCR-positive ticks (from Dorrigo) were equivocal. Further studies are required to demonstrate the sporozoite stage of the parasite within *H.longicornis* and to confirm transmission of the various *T.orientalis* genotypes from *H.longicornis* to cattle.

Passive*/* mechanical transfer of infection. To demonstrate the passive (mechanical or horizontal) transfer of infection, blood from a clinically infected donor was cryopreserved or freshly collected prior to inoculation into uninfected calves.*T.orientalis* infection can be transmitted by as little as 0.1ml blood from infected to naïve animals. Although this mode of transmission (of piroplasms in red cells) does not appear to result in significant disease,

parasitaemia can persist for at least 18 months, allowing for low-grade carriers of the parasites to occur within herds. This raises the possibility of transmission through husbandry practices and the likelihood that biting arthropods can act as mechanical vectors as distinct from obligate definitive hosts. Lice collected from one of the infected calves also tested positive for Theilerial DNA and passive transfer of infection from infected lice had been confirmed previously in Japan. Extrapolating our results would indicate that clinical disease arising from transmission of these haploid piroplasms by mechanical means (husbandry procedures, biting arthropods, colostrum and across the placenta) is unlikely. It is more likely that the sexual reproduction which occurs after blood feeding in the definitive host (tick) is necessary to generate and maintain the virulence of the parasite and its capacity to cause clinical disease and deaths. However, mechanical transmission might help to explain the epidemiology of the rapid spread of *T.orientalis* and associated clinical outbreaks in southern Australia and elsewhere. Since recipients in this study remained PCR-positive for over 15 months, more monitoring of recovered cattle in the field is required to establish whether sterile immunity is generated and if not, whether the disease can recrudesce under stress (e.g. subsequent calvings). Cattle mechanically infected by inoculation of infected blood may remain infective for ticks. However, it remains to be determined whether such "persistently-infected" calves are immune to tick challenge in the field.

In this study with limited numbers of animals, colostral transmission was not demonstrated. Maternal antibodies to *T.orientalis* could be detected in several newborns from dams with titres in colostrum. Transplacental transmission has been shown to occur at very low levels. From the outcomes from blood transmission, both routes are unlikely to generate clinical disease and account for the mortalities observed in young calves. In this respect, the transmission of immune defences from dam to calf via colostrum seems to be poor, as mortalities amongst young calves at 6-8 weeks of age is commonly reported from endemic areas. However, even if colostrum were to confer some protection against disease, the decline in maternal antibodies would be effectively complete by 4 weeks of age, leaving time for tick-borne infestations to occur and clinical signs to develop within 2 weeks to kill calves.

Industrial implications and further research. This research project has established the basis for active and passive transmission of *T.orientalis* infection. However, active transmission of the clinical disease with *H.longicornis* requires confirmation. Several other aspects of the disease epidemiology are also needed to confirm the most appropriate management and stock movement procedures. These include:(1) whether immunity is complete (no carrier state) after recovery from clinical disease, as persistent infection appears to occur after passive transfer of *T.orientalis;* and, (2) the impact of the carrier state for ongoing tick infection, immunity to tick challenge, stock agistment strategies and recurrence of clinical disease under stress.

Currently, prevention of the development of clinical disease requires either tick control , vaccination or chemotherapy. To evaluate each of these approaches, a standardised infective inoculum containing viable sporoziotes is needed for challenge of vaccinated animals or to test therapies.

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1 Background

1.1 Theileria in Australia

Theileria are blood-borne intracellular protozoal parasites belonging to the phylum *Apicomplexa.* Nomenclature has been a problem. The more benign forms of bovine *Theileria* have been referred to as *T. buffeli* in Australia, *T. sergenti* in Japan and *T. orientalis* in many other locations (Fujisaki, Kawazu et al. 1994). Serological and morphological comparisons showed all of these isolates to belong to one species, named as *T. orientalis* (Uilenberg, Perie et al. 1985). Another comparison of *T. sergenti/buffeli/orientalis* by Gubbels, *et al*. (2000) using criteria of morphology, vector specificity, pathogenicity and sequences of the 18S small subunit ribosomal RNA or major piroplasm protein also confirmed the various isolates as the same species and suggested the name *Theileria buffeli.* This supported the name proposed by (Callow 1984) and others (Stewart, Uilenberg et al. 1996).

Differences in the subgeneric level of the vector ticks and tick transmission experiments also support separation of *T. sergenti* (Fujisaki, Kawazu et al. 1994). For example, the Australian tick *H. longicornis* could transmit only *T. sergenti* but could not transmit *T. buffeli,* whereas Japanese *H. longicornis* could transmit both (Fujisaki, Kawazu et al. 1994). In addition, serological comparisons of piroplasm antigens of *T. sergenti, T. buffeli* and *T. orientalis* using ELISA and Western blotting in combination with 2D-PAGE also found dissimilarities in proteins of 33-kDa of *T. sergenti* and the 32 and 34-kDa of *T. orientalis/buffeli,* suggesting that *T. sergenti* should be separated from *T. orientalis* and *T. buffeli* (Kawazu, Sugimoto et al. 1992).

Despite the taxonomic discussions, the name *Theileria orientalis* is now widely applied to the species present in Australia, New Zealand and throughout Asia. It is now appreciated that *T. orientalis* can be separated into several genotypes, namely, type 1 (Chitose), type 2 (Ikeda), type 3 (Buffeli), types 4-8 (Islam, Jabbar et al. 2011, Kamau et al 2011) and types N1-N3 (Khukhuu, Lan et al. 2011). The various genotypes have been associated with varying severity of clinical signs, and may account for the recent epidemic of severe disease and deaths in Australia (Kamau et al 2011). Analysis of the MPSP gene, has identified eleven distinct *T. orientalis* genotypes (Sivakumar et al., 2014). Of these genotypes, Type 2 (Ikeda), and to a lesser extent, Type 1 (Chitose), are typically found in association with clinical disease (Kamau et al., 2011). A prevalence study of genotypes detected in New South Wales showed that Ikeda accounted for 46%, Chitose 46% and Buffeli 17.9% of infections (Kamau et al 2011).

The genotypes complicate blood smear diagnosis, such that PCR is used for definitive diagnosis and has great diagnostic (DSn) and analytical sensitivity. PCR testing for p32 gene DNA has been shown to be much more sensitive than clinical pathology in detecting *T. orientalis* infections (Eamens, Gonsalves et al. 2013). Around 90% of smear positive samples were positive in the p32 and Ikeda assays and the p32 PCR detected 33/69 (48%) of smear negative samples as positive for *T. orientalis*, and 39/69 (57%) of smear negative were confirmed to contain Ikeda (Eamens, Gonsalves et al. 2013). The DSn of PCR was found to be approximately 4.5 parasites ml^{-1} of blood, or equivalent to a parasitaemia of 0.00009% (Tanaka, Matsuba et al. 1992, Baek et al 2003).

Previously considered a benign parasite in Australia, outbreaks of clinical disease resulting from *Theileria orientalis* genotypes have been reported in Australia since 2006. Since this time, outbreaks have become widespread in south-eastern Australia, resulting in significant adverse impacts on local dairy and beef industries. Clinical signs of *T. orientalis* are mostly associated with the sequellae from anaemia. These signs can include depression, weakness, anorexia, pale mucous membranes, lymph node swelling, tachypnoea, tachycardia, dyspnoea, pneumonia, jaundice, late-term abortions, dystocia, still births, pyrexia, metritis, and mortality (Izzo, Poe et al. 2010, Islam, Jabbar et al. 2011). The early events following infestation from the vector is not known, but is presumed to involve schizont replication in the local lymph node before fever occurs coincident with detection of piroplasmic stages in erythrocytes around 7-9 days after infestation.

1.2 Approaches to examine transmission

1.2.1 Tick Vectors.

The method of introduction of *Theileria* to Australia is uncertain and may have occurred with the introduction of *Haemaphysalis longicornis* ticks into Australia (Seddon 1952). Transmission experiments involving *Theileria* in Australia occurred prior to clinical outbreaks of the disease. The vectors for *T. orientalis* Ikeda genotype have not been determined in Australia. The ticks *Rhipicephalus australis* (syn. *Boophilus microplus*) and *Haemaphysalis bispinosa* were once implicated as vectors in Australia (Seddon 1952), but this is no longer the case (Riek 1982). Many attempts to obtain transovarial and stage to stage (transtadial) transmission with *B. microplus* were not successful despite feeding larval, nymph and adult stages of ticks on animals displaying parasitaemia (Callow and Hoyte 1961, Riek 1982). While *R. australis* is unlikely to be a vector in Australia, other tick species have been demonstrated to transmit the infection. An earlier study showed *H. longicornis* is a highly successful vector for *T. buffeli*, as is *H. bancrofti,* while *I. holocyclus* and *Amblyomma triguttatum* have failed to transfer infection (Riek 1982). In Queensland, *H. bancrofti,* and *H. humerosa,* but not *H. longicornis* were shown to be vectors (Stewart, de Vos et al. 1987).

Experimental transmission studies in Japan employing the Japanese Ikeda genotype of *T. orientalis* with *H. longicornis* sourced from both Australian and Japanese populations were successful. In separate experiments, the native Australian wallaby tick, *H. bancrofti*, was shown to transmit both *T. orientalis* Ikeda (Fujisaki, Kawazu et al. 1994), and a strain of *T. orientalis* sourced from Queensland and presumed to be the Buffeli type “Warwick strain" (Stewart, Devos et al. 1989)**.**

Haemaphysalis longicornis, a known vector tick for *T. orientalis* internationally, has a wide host range, and a distribution along the coastal strip from south east Queensland to the Victoria border with rare occurrences in other locations in Victoria (Roberts 1970). It has also been reported in locations in south west Western Australia (Besier and Wroth 1985). It is a three host tick with larva, nymph, and adult engorging for approximately 7 days before dropping from the host (Cane 2010). Female ticks lay up to 2000 eggs in late spring and early summer and these hatch in 60-90 days depending on environmental conditions. Larvae have been shown to survive up to 217 days, nymphs 263 days and adults 249 days without feeding (Cane 2010). Most adults are seen in January and February. Reproduction is via parthenogenesis, making male adults rarely seen.

1.2.2 Mechanical transmission

Unequivocal evidence for mechanical transmission of *T. orientalis* is lacking. It has been hypothesised that mechanical transfer of theilerial piroplasms may occur from vaccination needles and the proboscis of biting insects (Heath 2013). There is limited evidence to support this, however a Japanese study found that a species of Tabanidae (*Tabanus trigeminus)*, under "certain" (unspecified) conditions can mechanically transfer *Theileria*; (cited in (Fujisaki, Kamio et al. 1993) and Onoe, Sugimoto et al. (1994). Fujisaki (1993) reported that splenectomised calves acquired *T. orientalis* Ikeda from lice previously fed on infested hosts (Fujisaki, Kamio et al. 1993).

1.2.3 Vertical transmission

Vertical transmission of *T. orientalis* has also been confirmed where foetuses or calves from 6 infected heifers were PCR-positive for the parasite (Baek *et al* 2003). Transfer from dam to calf through colostrum is another possible mode of transmission but has not been specifically investigated. A separate study observed possible prenatal infections of *T. orientalis* where 5 out of 100 blood samples of 1 or 2 day old calves were positive for *Theileria* with parasitaemias between 0.01 and 0.06% (Onoe, Sugimoto et al. 1994). However, this may have occurred through ingestion of sanguinous colostrum as well.

2 Project objectives

2.1 Identification of the vector(s) for transmission of *Theileria orientalis* .

2.2 To establish the conditions for passive transfer of infection

2.3 To establish the seasonality of transmission related to clinical disease.

2.4 Classification of haematophagous arthropods in Victoria.

3 Methodology

3.1 Collection and identification of biting arthropods

3.1.1 Ticks

Some 220 ticks were collected from a variety of livestock and wildlife hosts over a large geographical region of Victoria by local veterinary practices, wildlife carers, farmers and members of the public. *H.longicornis* were also sourced from Dorrigo NSW, collected Dr Chris Shirley, local veterinarian.

Preliminary identification of ticks was undertaken morphologically using identification charts (Roberts 1970) and photographed. All ticks collected were subsequently transported on dry ice to the laboratory for molecular identification. DNA was extracted from the tick legs using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. The

mitochondrial cytochrome oxidase I (COI) genes were amplified using the metazoan "barcoding" primers of Folmer (Folmer, Black et al. 1994).

3.1.2 Haematophagous dipterids (flies and mosquitos)

Haematophagous flies were collected during suitable weather during summer 2013/14, autumn 2014 and the summer of 2014/15 in locations within 1kmof outbreak farms. Two Nzi traps (www.nzitrap.com) were set up using dry ice and 1-Octen-3-ol as an attractant. A total of 4 (UV) light traps (Australian Entomology Supplies, Model PR101) were used for the collection of mosquitoes.

3.1.3 Lice

Hundreds of the sucking louse *(Linognathus vituli)* were collected from two calves that had been mechanically infested (below) on the experimental property in Fernbank, Victoria (37°48'21.9"S 147°19'41.7"E). Lice were collected with a flea comb and identified morphologically.

3.2 Testing collected arthropods for T. orientalis

Ticks grouped by species or phylogenetic cluster, and where possible by host and location, were dissected for quantitative PCR (qPCR) analysis by removal and collection of the capitulum and the anterior portion of the scutum. Tick tissue was pooled in groups of 1-10 samples depending on phylogenetic group but also on tissue volume so as not to exceed a total of 25 mg of tissue per pool.

For mosquitoes and tabanid flies, tissue from the abdomen and thorax was extracted. Mosquitoes were also tested in 10-15 mg pools each containing around 100 mosquitoes. Mosquitoes and march flies (*Dasybasis* sp.) were batch tested. Thirteen pools of mosquitoes were tested.

Seven pools of lice, each containing 25 lice from each calf were tested. Hundreds of other lice from the same donor were tested in a bulk pool. Each pool of lice was homogenized prior to extraction with a sterile microfuge pestle. DNA extraction was performed as described above for the tick legs.

Quantitative PCR (qPCR) was performed at EMAI, Menangle, NSW using a validated qPCR assay for *T. orientalis* detection and genotype differentiation as described previously (Bogema, Deutscher et al. 2014). This test determines the genotype of *T.orientalis* present (eg, buffeli, chitose or ikeda) and the proportion of these genotypes in the infestations at each time point.

3.3 Mechanical transfer

Thirteen bucket reared Holstein-Friesian steers aged 6 months were sourced from a commercial dairy herd near Bairnsdale in Victoria, and were separated from the dam within 24 hours of birth after receiving adequate colostrum They were immediately blood tested confirm a negative status for *T. orientalis*, and moved to the study location (37º44'03.6"S 147º39'31.6"E), near Bairnsdale in Victoria.

A recumbent Angus steer exhibiting clinical signs of theileriosis (tachypnoea, tachycardia, pale mucous membranes, weakness), had a packed cell volume of 8% and piroplasms in a blood smear. Diagnosis was confirmed by PCR and the genotype was confirmed as *T. orientalis* Ikeda. The quantitative PCR (qPCR) of this stabilate was 1678.99 femtograms/microlitre (fg/µL) (251,849 gene copy number) for universal *T. orientalis,* and 1570.78 fg/µL Ikeda genotype. This parasitaemia is considered in the clinically relevant range (Bogema, Deutscher et al. 2015).

Using a CPDA-1 single blood-pack Unit (Fenwal International Inc.), 250 ml of blood was collected from the jugular vein of this steer and cryopreserved.

Group 1 (Calves H1, H2, H3 - high) were inoculated intravenously with 20 ml of inoculum containing 10 ml of the **cryopreserved**, infected blood. Based on the gene copy number of this inoculum, the total number of parasites transferred was approximately 2.5 billion. Group 2 (Calves M1, M2, M3 - medium) each received 1 ml of **fresh** blood (approx.154.4 million parasites). This fresh blood was collected in syringes containing 0.14 ml of anticoagulant (CPD-1) drawn up and mixed with one ml of blood collected from the jugular vein of the positive control calf. Group 3 (Calves L1, L2, L3 - low) each received 0.2 ml of inoculum, containing 0.1 ml **cryopreserved** blood (approx. 25.2 million parasites). Group 4 contained 3 negative control calves and the positive donor calf. The 3 negative control calves acted as sentinel animals for active transmission by ticks and other possible vectors.

To detect theilerial infection, blood was collected from the tail veins of all calves into 5 ml EDTA blood tubes three times weekly for 13 weeks. Collected blood was frozen, and sent in bulk to EMAI, Menangle to be tested via qPCR for the presence of *T. orientalis* and genotype differentiation.

3.4 Colostral transmission

Thirty cows from a commercial dairy herd within the endemic region of Bairnsdale, Victoria were sampled when they calved over a 3-week period in July 2015. Colostrum was collected from each quarter into a single sterile container within 24 hours of giving birth. The samples were immediately frozen and transported for testing by qPCR in bulk for *T. orientalis* and by ELISA for anti-Theilerial antibodies*.* Blood samples were collected from all dams who had colostrum tested 3-6 weeks after giving birth. The calves of these cows, who received colostrum were tested at the same time as the cow when their ages ranged from 3 to 6 weeks. As the male calves had been sold, a total of 19 female calves were available for testing.

3.5 Transplacental transmission

The same commercial dairy herd was used to investigate transplacental transmission. Over a 2 week period, 5 ml of blood was collected from thirty cows and their calves. Samples were then frozen and sent for qPCR analysis from the EDTA blood samples while ELISA antibody testing was performed on the serum from separate clotted samples. ELISA assays were conducted at EMAI, Menangle, NSW, using MPSP antigen. Results were expressed as an ELISA ratio (ER: mean OD test serum/mean OD of the negative control serum). Sera with an ER < 2 were considered negative and an ER ≥ 2 as positive. These results were shown previously to give a specificity of 98% and sensitivity of 67% in comparison to PCR testing (Eamens and Jenkins 2013).

4 Results

4.1 Vector Studies

A total of 81 tick COI barcodes were identified; 56 from ticks of the *Haemaphysalis* genus, 18 from *Ixodes* spp. and 7 from *Bothriocroton* spp. Thousands of lice were collected and identified as the long-nosed sucking cattle louse *Linognathus vituli.* Approximately seven thousand mosquitoes were collected on outbreak farms or within close proximity. The mosquitoes identified include; *Aedes camptorhynchus, Aedes notoscriptus, Coquillettidia linealis, Culex australicus,* and *Culex molestus*. The biting fly collected was the March fly *(Dasybasis sp.)*. Only 28 of these flies were collected and tested for *T. orientalis.*

Amongst the ticks examined, only *H. longicornis* was found to be positive for theilerial DNA. Ticks sourced over a large geographical area were found to harbour all 3 genotypes of *T. orientalis* examined (Ikeda, Chitose and Buffeli). The Ikeda genotype was found in all pools of ticks testing positive for *T. orientalis* (see Hammer et al., 2015).

Of the 13 pools of mosquitoes tested, one was weakly positive for *T. orientalis* (41 GC/µL), with only the Buffeli genotype identified over the limit of detection of the assay. Pool testing of the remaining mosquitoes also returned weakly positive reactions (20 GC/µL) for *T. orientalis* with no genotypes exceeding the limits of detection for the respective assays. Biting flies (n=28) were found to be negative for *T. orientalis* all genotypes, while all 14 pools of *Linognathus vituli* lice tested positive for *T. orientalis* Ikeda*.* Blood collected from the two lousy calves were positive for *T. orientalis* with infection intensities of 89.07 fg/µL (Calf 1), and 143.85 fg/µL (Calf 2). The average infection intensity from the batches of lice from calf one was 0.33 fg/µL (49.50 gene copies) for universal *T. orientalis* and 1.14 fg/µL (171.72 gene copies) for the Ikeda genotype. The average intensity from the batches of lice from calf two was 0.24 fg/µL (36.44 gene copies) for universal *T. orientalis* and 0.47 fg/µL (46.22 gene copies) for the Ikeda genotype, the Ikeda probe being more senstive. The infection intensities for Ikeda correspond to low range. All lice tested negative for the Chitose and Buffeli genotypes.

Blood testing for this part of the study also highlighted the prevalence of the genotypes in this herd. Of the 63% of cattle that tested positive for universal *T.orientalis*, the Ikeda genotype was evident in 84%, Chitose in 37%, and Buffeli in 32%. Mixed infections were seen in 36% of positive cases. Of the mixed infections 29% were Ikeda-Chitose, 14% Chitose-Buffeli, 14% Ikeda-Buffeli, and 42% had mixture of Ikeda-Chitose-Buffeli.

4.2 Mechanical transfer

Calves in the high, (10 ml of cryopreserved blood) medium (1 ml fresh blood) and low dose groups (0.1ml of cryopreserved blood) groups became positive for Theilerial DNA at 28 days (4 weeks), 41 days (6 weeks) and 66-98 days, respectively, following transfusion. Calves have **remained positive for more than 15 months** after infection to date. However, it was noted that none of the 9 recipients exhibited overt clinical signs of disease nor suffered anaemia.

4.3 Transplacental transfer

Around 63% of cattle (19/30) tested positive for *T. orientalis* with Ikeda genotype accounting for 84%, Chitose 37% and Buffeli 32% of infections. Thirty-six percent of infections were mixed infections. Out of the PCR positive cattle, only 2 dams and one calf tested ELISA positive for antibodies. Only one calf was anaemic from all of the samples collected, and this calf was positive for antibodies and had a mother also testing positive for both theilerial DNA (qPCR) and antibodies for *T. orientalis.*

4.4 Colostral transfer

Sixteen of the 30 dams tested positive for *T. orientalis.* Of these 16 Dams, 67% were positive for Ikeda genotype only, 25% had a mixed infection of Ikeda and Buffeli, and one dam tested for Chitose only.

Of the 30 colostrum samples collected from the Theileria-positive dams, 4 tested positive, and a further 11 had amplification but was below the diagnostic threshold of the assay. In addition, 6 samples of colostrum tested positive for antibodies to MPSP in ELISA. Of the 6 calves that received antibodies in colostrum, only one tested positive for antibodies. Of the 4 samples of colostrum that tested positive for *T. orientalis,* no evidence of transmission to calves was demonstrated. No calves tested positive for *T. orientalis.*

4.5 Active transfer – Salivary gland analysis

Since insufficient ticks could be collected from endemic regions to make stabilate to attempt active transfer of infection with salivary glands, 50 ticks collected in Feb 2016 from cattle in Theileria-endemic herds in each of Bairnsdale and Dorrigo were frozen. Twelve engorged ticks (chosen to allow maximum time for sporozoite development) were sliced in half longitudinally. One half was kept frozen for PCR and the other was placed into Bouins fixative for histopathology.

The ticks were examined at EMAI. Tests showed that 2/12 Bairnsdale ticks and 10/12 Dorrigo ticks were PCR positive, indicating either piroplasmic blood meal (as detected earlier) or sporozoite stages. However, sectioning of the fixed ticks indicated that salivary glands in engorged ticks were severely compressed and very difficult to delineate with any certainty. Only 1 was found with intracytoplasmic inclusions in salivary glands, but this was equivocal.

5 Discussion

5.1 Vectors of T.orientalis

Prior evidence for the transmission of *T. orientalis* by Haemaphysalis ticks in Australia has been contradictory, but results from this and previous studies provide strong circumstantial evidence for the role of *H.longicornis*. *H.longicornis* ticks sourced from both Japan and Australia have been shown experimentally in Japan to transmit the *T.orientalis* Ikeda genotype trans-stadially (Fujisaki, Kawazu et al. 1994). The *H.longicornis* barcoding sequences obtained in this study suggest that the *H. longicornis* populations in Eastern Victoria are relatively homogenous and are closely related to those in Asia. Additionally, the

known range of *H. longicornis* in Australia closely mirrors areas in which outbreaks of clinical theileriosis have occurred, including an isolated population of this species in the south west of Western Australia. This information and the detection of *T.orientalis* Ikeda DNA in ticks from various regions in Victoria support the premise that *H. longicornis* is a likely vector for *T. orientalis* Ikeda in Australia. Based on previous studies, the ability of *H.longicornis* to transmit *T.orientalis* Buffeli is less certain (Stewart, de Vos et al. 1987). It is noteworthy therefore, that the Buffeli genotype (as well as the Chitose genotype) was detected within *H. longicornis* ticks along with the Ikeda genotype. More extensive analysis of the proportions of the *T.orientalis* genotypes within individual ticks would be of future interest to determine whether particular genotypes are selected during the tick phase of the parasite's lifecycle.

This study also revealed *H. longicornis* as the major tick species found on cattle in Victorian herds suffering recent clinical outbreaks, further implicating this species as a likely vector of bovine theileriosis. However, attempts to provide conclusive evidence by active transmission with tick saliva or salivary glands were thwarted by lack of ticks in season 2014. Simple stabilate could not be used because of the ease of mechanical transmission with piroplasms. Additional sectioning of infected ticks to demonstrate sporoziote development in salivary glands were equivocal due to the damage to salivary gland architecture in fully engorged ticks. Such a study in future should be undertaken in nymphs or partially engorged adults.

Other Haemaphysalis and Ixodes spp. have recently been implicated in transmission of *T.orientalis* in the Eastern Hokkaido and Okinawa prefectures via molecular screening of tick species for the presence of the parasite (Yokoyama, Sivakumar et al. 2012). Interestingly, we did not detect theilerial DNA in any other tick species. New Zealand has experienced very similar outbreaks of *T.orientalis* linked to the Ikeda genotype since 2012 (Watts, Playford et al. 2015). While there are a number of potential vectors for transmission of *T.orientalis* in Australia, New Zealand only has one livestock-infesting tick; *H.longicornis* (Heath 2015), making this the likely vector in New Zealand, and also increasing the likelihood of *H.longicornis* being the vector in Southern Australia.

5.2 Mechanical transfer of infection

The results of this study has shown that Theilerial infections can be readily transferred by infected blood. Infections detectable by PCR occurred after inoculation of fresh or cryopreserved, infected, bovine blood. The relationship between inoculated blood volume and time to patency clearly indicated the inverse dose- response and that the cryopreservation process preserved the viability of the piroplasms. From the level of donor parasitism (around 2%), the transfer of around 10^7 parasitised erythrocytes (in 0.1 ml) can establish a parasitosis with sufficient quantum to be detected and graded by qPCR as a low-level infection. [The diagnostic threshold or sensitivity of the test used in this trial was 30 gene copies per microlitre of blood; equivalent to 30 parasites per microlitre of blood]. In this study, transplacental transmission was not demonstrated in 19 PCR-positive dams to their newborn calves. However, Onoe et al. (1994) reported *Theileria [sergenti*]-like organisms in 1-2 day old calves, while Baek et al, 2003 detected intra-uterine transmission of *T.sergenti*. A recent, more extensive study by Swilks *et al* also found evidence of very low rates of transplacental infection. It was noted during the conduct of this project that calves generally died of *T.orientalis* around 6+ weeks of age. However, based on our findings that mechanical transfer (even through colostrum) would not cause clinical disease, these calf mortalities should have arisen from tick infestation after birth.

Colostral transmission was not demonstrated in this project. However, the small numbers of cattle studied (11), could not exclude the colostral route, as 1 of 2 calves receiving anti-MPSP antibodies in colostrum, exhibited positive serum titres of maternal origin at 3 weeks of age. More samples should be tested to consolidate this result which is important for the epidemiology of the disease.

Piroplasms could be transferred from one animal to another iatrogenically when vaccinating a mob of cattle, or giving medications to cattle by using the same needle between animals. Other examples of blood transfer between cattle could include contaminated castration knives, ear notching procedures, as well as injury sustained during yarding and transport of cattle. Since *T.orientalis* can be transmitted though the lowest dose examined in this trial (0.1 ml), the risk of mechanical transmission through husbandry practices cannot be excluded. The risk of iatrogenic transmission would depend on the volume and parasitaemia of the blood transferred and on the ability of the parasite to survive outside a host before being inoculated into a susceptible animal.

Two features of the mechanical transfer are important. Firstly, recipients did not develop clinical signs of parasitism, and secondly, ALL have remained PCR-positive for more than 15 months, which implies that cattle do not generate sterile immunity following mechanical transmission. These results have obvious implications for immunity and control, such that more monitoring of recovered cattle in the field is required to establish whether sterile immunity is generated and if not, whether the disease can recrudesce under stress (e.g. subsequent calvings). In addition, calves mechanically infected by inoculation of infected blood may remain infective for ticks. In Japan, *H.longicornis* ticks were successfully infested when fed on calves which had been infected from lice from a previously infected animal. This aspect of the life cycle epidemiology needs to be confirmed for control of the disease. In relation to the lack of clinical signs after mechanical transmission, the sexual phase of the *T.orientalis* lifecycle occurs within vector ticks, allowing genetic recombination to occur. This may be necessary for the maintenance of virulence and the ability to cause clinical disease. However, since the parasitosis persists in the recipient, mechanical transmission may help to explain the rapid recent spread of the parasite over large geographical areas in Australia. Equally, calves infected mechanically and remaining subclinically infested with piroplasms may be protected from tick-borne disease.This also needs to be assessed as a potential "vaccine".

Haematophagous insects; including flies, lice and mosquitoes as well as non-vector ticks could have the potential to transmit Theileria. Regurgitation of part of a previous blood meal or passive transfer of blood on mouth parts are possible modes of transmission depending on both the level of parasitaemia in the donor and the cumulative volumes inoculated or transferred by the biting arthropods. We detected DNA of *T.orientalis* in at least one pool of 100 mosquitoes (and 2 more were weakly positive) captured in close proximity to herds experiencing clinical cases of theileriosis. However, PCR detection of infection was demonstrated in the louse, *Linognathus vituli* from mechanically infected donors. In Japan, lice have been infected from calves with *T.orientalis* (from tick stabilate) and then used to transfer infection to naïve calves, from which ticks subsequently became infected after application (Kamio, Fujisaki et al. 1989). Given the uptake of infection by lice in this study, the Japanese work implies that similar outcomes would be expected in Australia.

In this study, the number of haematophagous flies caught on the outbreak farms was too low be conclusive. We did not detect *T.orientalis* in biting flies in an outbreak region, but further research is warrented to exclude any major role in mechanical transmission.

6 Conclusions/recommendations

This project presents the first data identifying DNA from *T.orientalis* in *H.longicornis* from herds with recent outbreaks of clinical theileriosis. This adds to the body of international evidence for its role as an intermediate host in the life cycle and transmission of the parasite. Further studies are required to demonstrate the sporozoite stage of the parasite within *H.longicornis* and to confirm transmission of the various T.orientalis genotypes from *H.longicornis* to cattle.

T.orientalis can be transmitted by low volumes of blood transferred from infected to naïve animals. Although this mode of transmission does not appear to result in significant disease, parasitaemia can persist and allow for low-grade carriers of the parasites to occur within herds. This raises the possibility of iatrogenic transmission through husbandry practices and the likelihood that biting arthropods can act as mechanical vectors as distinct from obligatory intermediate hosts. However, our results would indicate that clinical disease is unlikely. Mechanical transmission might help to explain the epidemiology of the rapid spread of *T.orientalis* and associated clinical outbreaks in southern Australia and elsewhere. Since recipients in this study remained PCR-positive for over 15 months, more monitoring of recovered cattle in the field is required to establish whether sterile immunity is generated and if not, whether the disease can recrudesce under stress (e.g. subsequent calvings). In addition, calves mechanically infected by inoculation of infected blood may remain infective for ticks. However, it reamins to be examined whether such "persistently-infected" calves are immune to tick challenge in the field.

Colostral transmission could play a role is disease transmission, while transplacental transmission does not seem likely. Both are unlikely to generate clinical disease and account for the mortalities observed in young calves. This study is the first to report *T.orientalis* in colostrum, and also the first to show antibodies in colostrum and their transfer to feeding calves. However, the transmission of immune defences from dam to calf via colostrum seems to be poor, as mortalities amongst young calves at 6-8 weeks of age is commonly reported from endemic areas. The decline in maternal antiboides would be effectively complete by 4 weeks of age, leaving time for tick-borne infestations to occur and clinical signs to develop within 2 weeks to kill calves.

7 Bibliography

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