Temporal dynamics and subpopulation analysis of *Theileria orientalis* genotypes in cattle

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Abstract:

In Australia, outbreaks of clinical theileriosis caused by *Theileria orientalis* have been largely associated with the Ikeda genotype which can occur as a sole infection, or more commonly, as a mixture of genotypes. The most prevalent genotype, Chitose, frequently co-occurs with type Ikeda, however the role of this genotype in clinical disease has not been clearly established. Furthermore, the dynamics of individual genotypes in field infection of cattle have not been examined. In this study we developed quantitative PCR (qPCR) and genotyping methods to examine the role of the Chitose genotype in clinical disease and to investigate the temporal dynamics of *T. orientalis* Ikeda, Chitose and Buffeli genotypes in naïve animals introduced to a *T. orientalis*-endemic area. Analysis of the major piroplasm surface protein (MPSP) genes of Chitose isolates revealed the presence of two distinct phylogenetic clusters, Chitose A and Chitose B. A genotyping assay aimed at determining Chitose A/B allele frequency revealed that the Chitose A phylogenetic cluster is strongly associated with clinical disease but nearly always co-occurs with the Ikeda genotype. qPCR revealed that the Chitose genotype (particularly Chitose A), undergoes temporal switching in conjunction with the Ikeda genotype and contributes substantially to the overall parasite burden. The benign Buffeli genotype can also undergo temporal switching but levels of this genotype appear to remain low relative to the Ikeda and Chitose types. Interplay between vector and host immunological factors is presumed to be critical to the population dynamics observed in this study. Genotypic switching likely contributes to the persistence of *T. orientalis* in the host.

1. Introduction:

Theileria orientalis is a tick-borne haemoprotozoan parasite of cattle and buffalo which can cause disease during the intraerythrocytic (piroplasm) stage of its lifecycle. The major clinical manifestations of the disease are anaemia, jaundice, lethargy, tachycardia and late-term abortion in pregnant animals (Eamens et al., 2013c; Izzo et al., 2010; Kamau et al., 2011). *T. orientalis* infections can remain subclinical, although stress may cause the disease to recrudesce (Kamau et al., 2011; McFadden et al., 2011) and animals are infected long-term, perhaps for life (Kubota et al., 1996). The mechanisms for the persistence of *T. orientalis* in the host have not been elucidated. Currently, eleven genotypes of *T. orientalis* have been identified, types 1-8 and N1-N3 (Jeong et al., 2010; Khukhuu et al., 2011; Sivakumar et al., 2014). These genotypes are defined based on the sequences of the major piroplasm surface protein (MPSP), an immunodominant antigen expressed during several phases of the parasite's lifecycle (Sako et al. 1999). Of the eleven MPSP genotypes, Chitose (Type 1) and Ikeda (Type 2) have been associated with clinical disease (Cufos et al., 2012; Eamens et al., 2013b; Eamens et al., 2013c; McFadden et al., 2011); while the Buffeli type (Type 3) and its phylogenetic sister group, Type 5 are considered benign (Kamau et al., 2011a). The clinical relevance of the remaining types has not been clearly elucidated; however recent clinical outbreaks of disease in India appear to be related to the presence of Type 7, a phylogenetic relative of the Ikeda type (Aparna et al., 2011). In Australia, the Ikeda, Chitose and Buffeli genotypes have thus far been identified, with Type 5 also occurring in infected cattle, albeit with low prevalence.

T. orientalis infection often occurs as a mixture of genotypes. Recent outbreaks of clinical theileriosis in Australasia have been linked to infection with the Ikeda genotype. In one study, this genotype was found to be present in clinical cases as a sole or mixed infection

(Eamens et al., 2013c), but most commonly co-occurred with the Chitose genotype. In contrast to the Ikeda genotype, the Chitose genotype was rarely found to be associated with disease when present as a sole infection (Eamens et al., 2013c); however other studies have suggested that the Chitose genotype may directly cause clinical disease (McFadden et al., 2011). While the Buffeli genotype is considered benign and does not cause disease when present as the sole infection, it can also occur in combination with the Ikeda and/or Chitose genotypes in clinical cases (Eamens et al., 2013c).

In this study, we used quantitative PCR methods to investigate associations between phylogenetic subpopulations of the Chitose genotype and disease, and to monitor the temporal dynamics of the Ikeda, Chitose and Buffeli genotypes of *T. orientalis* in a group of naïve animals introduced to a *Theileria*-endemic area.

2. Methods:

2.1. Samples

Samples used in this study were derived from diverse geographic regions of Australia, within the states of New South Wales (NSW), Victoria, Queensland and South Australia. All *T. orientalis* positive samples (n = 137) were derived either from herds with clinical theileriosis cases (n = 89) or from herds with subclinical infections at the time of sampling (n = 48) and were characterised in a previous study as Chitose positive infections (Bogema et al., 2015; Eamens et al., 2013a; Eamens et al., 2013b; Eamens et al., 2013c). Negative samples were sourced from an area where *T. orientalis* was not enzootic and were confirmed negative by conventional PCR (Bogema et al., 2015). *T. orientalis*-negative samples sourced from cattle known to be infected with *Babesia bigemina* or *Babesia bovis*, causative agents of the clinically similar disease, tick fever; or derived from cattle immunized with *Anaplasma*

centrale, were kindly provided by Dr Phil Carter at the Tick Fever Centre, Wacol (Bogema et al., 2015). Time-course samples were collected from a mob of 10 naïve Ayrshire cattle that had been introduced to an area on the mid-coast of New South Wales, Australia, where *T. orientalis* was known to be enzootic, and onto a property with a prior history of clinical theileriosis cases. Blood samples were collected immediately upon introduction of the cattle and approximately weekly thereafter for a period of 11 weeks. Immediately following blood collection, packed cell volume (PCV) was measured and blood films were prepared and stained with Diff-Quik (Australian Biostain, Traralgon, Australia). All samples were collected in vacuum blood tubes containing either EDTA or lithium-heparin. Blood was decanted into sterile sample tubes and stored at -20°C for later extraction.

2.2. DNA extraction

Purification of sample DNA was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) with a 100 µL starting volume of blood and a 100 µL elution volume. Negative extraction controls were included at a ratio of at least 1 per 20 DNA extractions as a quality control measure.

2.3. Phylogenetic analysis

Partial MPSP genes from the Chitose genotype were amplified as previously described (Eamens et al., 2013a; Zakimi et al., 2006) from selected samples derived either from herds with clinical theileriosis cases or from herds with subclinically infected animals. Amplicons were purified using the Qiaquick PCR purification kit (Qiagen) and were submitted to the Australian Genome Research Facility (AGRF) for Sanger sequencing. Contiguous MPSP gene sequences were compiled using Geneious vR7 (Biomatters, Auckland, New Zealand) and aligned with representative Chitose genotype sequences from GenBank using MUSCLE.

Sequences were trimmed prior to analysis, with a total of 703bp of sequence analysed. All sequences have been submitted to GenBank (Accession numbers pending). Phylogenetic analysis was performed within MEGA v6 using the neighbour joining, parsimony (maximum composite likelihood), and maximum likelihood (Tamura-Nei) methods with 1000 bootstrap replications. An MPSP gene sequence from the Buffeli genotype was used as an outgroup.

2.4. Primer and TaqMan probe design

Dual-labelled TaqMan hydrolysis probes were used for all genotype-specific qPCR assays and for the Chitose subpopulation analysis. Three specific qPCR assays were designed to target the three dominant Australian *T. orientalis* genotypes (Ikeda, Chitose and Buffeli). A fourth assay was designed to determine the dominant phylogenetic subpopulation (Chitose A or Chitose B) of the Chitose genotype. Each assay consisted of a probe and a forward and reverse primer set that were all genotype-specific. Each probe was 5′ labelled with a fluorophore (Table 1) and 3′ labelled with a non-fluorescent quencher (NFQ). In the case of the Chitose-specific qPCR, a degenerate design was used to account for a polymorphism between Chitose A and Chitose B subpopulations (Table 1). All probes contained an MGB moiety to confer additional probe specificity and were purchased from Life Technologies (Carlsbad, CA, USA). All primer and probe sequences are listed in Table 1. An *in silico* analysis was performed on all primers and probes by comparison with existing sequence data in GenBank. Each probe and primer set was designed to maximise detection of their respective subtypes.

2.5. Quantitative PCR (qPCR) and generation of plasmid standards

Plasmid DNA standards, reagents, equipment, consumables and thermal cycling parameters for qPCRs performed in this study were as described previously (Bogema et al., 2015) with primers and probes for the Ikeda, Chitose and Buffeli subtype assays as listed in Table 1.

The limit of detection (LOD) of each of these assays was defined as the limit where 95% of qPCRs were successful (Bustin et al., 2009). This was determined experimentally by testing 8 replicates of an equimolar mixture of Ikeda, Chitose (A+B) and Buffeli plasmid DNA at dilutions 1500, 150, 100, 50, 15, 5, 1.5 and 0.5 MPSP gene copies per µL (GC/µL), followed by Probit analysis.

The assay used for determining subpopulation ratios of the Chitose genotype was performed using the same method as the Chitose genotyping qPCR with the exception that the Chitose A and Chitose B probes were present in different proportions and labelled with different fluorophores (Table 1).

2.6. qPCR specificity

Probe specificity was tested by performing genotype-specific qPCRs with pairwise combinations of each primer set, probe and plasmid (ie. For Ikeda primers, probe/plasmid combinations Ikeda/Ikeda, Ikeda/Chitose, Ikeda/Buffeli, Chitose/Ikeda, Chitose/Chitose, Chitose/Buffeli, Buffeli/Ikeda, Buffeli/Chitose and Buffeli/Buffeli were tested). Specificity was also tested using 10 samples derived from *T. orientalis*-negative animals, or *T. orientalis*-negative animals known to be positive for *B. bovis, B. bigemina* or *A. centrale.* The performance of each assay in detecting its target genotype within clinical samples was also tested using 15 well-characterised (sequenced and/or tested with alternate assays) samples

known to be positive for one or more of the Ikeda, Chitose and Buffeli genotypes (Bogema et al., 2015; Eamens et al., 2013a).

2.7. Data Analysis

Analysis of qPCR data was performed as previously described (Bogema et al., 2015). Genotyping of selected samples were confirmed via Sanger sequencing at the Australian Genome Research Facility (AGRF) using sequencing primers detailed in Table 1. For the Chitose subpopulation assay, Chitose A/B ratios were determined by comparing to 7 duplicate ratio standards of 1.00:0.00, 0.95:0.05, 0.75:0.25, 0.50:0.50, 0.25:0.75, 0.05:0.95 and 0.00:1.00 Chitose A:Chitose B plasmid at 100 $fg.\mu L^{-1}$. Subpopulation ratio determination was performed by analysis of real-time PCR curve inflexion points as described in Chen et al., (2014). To obtain these, raw fluorescence outputs were normalized to a passive reference dye (ROX), baseline-subtracted and corrected for cross-talk using pure fluorophore calibration data. Calculated ΔRn values were then fitted to a non-linear sigmoid function using Prism 4.0 (GraphPad Software, La Jolla, CA, USA) and transformed fluorescence ratios (k') were calculated from inflexion points. Unknown Chitose A:Chitose B ratios were calculated from a standard curve of transformed fluorescence ratio (k') vs Chitose A allele frequency fitted to a non-linear function (Chen et al, 2014). A conservative upper allele frequency limit of 95% was used to define 'pure' (>95%) Chitose A or B alleles from mixed populations. Chitose A/B ratios from this analysis were used to sort Chitose positives into three groups, >95% Chitose A, mix Chitose A/B and >95% Chitose B. Correlations of Chitose A/B ratio with clinical disease and Ikeda prevalence were determined using Pearson's chi-squared test.

3. Results

3.1. *Sequence analysis reveals phylogenetic subpopulations within the Chitose genotype*

While the role of *T. orientalis* Ikeda in clinical theileriosis has been noted in several prior studies (Eamens et al., 2013c; Kamau et al., 2011; Perera et al., 2013), the role of the Chitose genotype is less clear. To investigate the Chitose genotype further, twenty two *T. orientalis* Chitose MPSP gene sequences derived from individuals within herds displaying clinical theileriosis cases, and from herds with subclinically-affected animals were compared with reference sequences from GenBank (Figure 1). Sequence analysis revealed two distinct phylogenetic clusters of *T. orientalis* Chitose (referred to here as Chitose A and Chitose B) that were consistently observed using disparate phylogenetic methods and were well-supported by the bootstrap analyses. These two phylogenetic clusters were characterised by a series of single nucleotide polymorphisms within the MPSP gene sequences (data not shown). There was slightly more sequence diversity within the Chitose B cluster (98.7-100% identity) than the Chitose A cluster (99.3-100% identity). The Australian Chitose sequences were polyphyletic, with sequences from both the Chitose A and B clades represented; however the sequences analysed from Queensland, where relatively few clinical outbreaks have occurred, were all of the Chitose B type. Futhermore, sequences belonging to the Chitose A cluster were all from New South Wales where clinical outbreaks have been occurring since 2006 and showed a close relationship with Chitose sequences from Japan. Of the Australian samples sequenced in this study, it was noted that all sequences from the Chitose A phylogenetic cluster (n = 7) were derived from herds with clinical cases. In addition, 3 Australian sequences (Foster3, Foster4 and Kempsey6) from a previous study (Kamau et al., 2011) were also derived from clinically-affected herds. All of the samples sequenced in this study from herds with subclinically infected animals (n = 9) belonged to the Chitose B cluster; however this cluster also included some sequences from herds with clinically-affected (n = 6) animals. Finally, all sequences within the Chitose A cluster had

been identified in prior studies as mixed infections, all co-occurring with the Ikeda genotype (Eamens et al., 2013a), while sequences from the Chitose B cluster derived from both cases of sole Chitose infection or infections of mixed genotype.

3.2. A phylogenetic subpopulation of the Chitose genotype (Chitose A) is strongly associated with both clinical disease and genotype Ikeda

To test the association of the Chitose A phylogenetic type with clinical disease, a genotyping assay based on the method of Chen et al., (2014) aimed at discriminating Chitose A and B subpopulations was used to examine 137 samples which tested positive for the Chitose genotype in prior studies (Bogema et al., 2015; Eamens et al., 2013a; Eamens et al., 2013c). For this analysis the transformed fluorescence ratio (k′) and allele frequencies of the Chitose A genotype (mixed with Chitose B) were compared. Using predetermined standard plasmid ratios, transformed fluorescence ratio (k') showed a non-linear relationship with Chitose A allele frequency (R^2 = 0.998; Figure S1). Allele frequencies determined from the blood samples are summarised in Table 2. Pearson's Chi-squared test indicated a strong association of clinical disease ($P < 0.0001$) and presence of Ikeda genotype ($P < 0.0001$) with samples that were predominantly Chitose A. Conversely, the same analysis indicated that high proportions (> 95%) of Chitose B were associated with the absence of clinical disease and negative detection of the Ikeda genotype. 100% of the samples with >95% of Chitose A allele were sourced from the state of NSW (Table 3). Additionally, the presence of Chitose A (> 95% + mixed) was found to have a significantly higher correlation with Ikeda than the presence of Chitose B ($P < 0.0001$). In addition, all samples sourced from Queensland contained >95% of the Chitose B type (Table 3).

3.3. *Temporal dynamics of genotype populations in T. orientalis*-infected cattle

To investigate the temporal dynamics of *T. orientalis* populations in field-infected animals, we developed specific qPCR assays for the three major genotypes of this parasite found in Australia, namely the Ikeda, Chitose and Buffeli genotypes. Analytical sensitivity statistics and the linearity of standard curves from these three assays are outlined in Table 4 and Figure S2. The specificity of primer-probe sets for each intended target was confirmed using plasmids containing Ikeda, Chitose (A+B) and Buffeli MPSP gene sequences at 100 $pg.\mu L^{-1}$ (Figure S3). In addition, 10 samples collected from cattle cPCR-negative for *T. orientalis* or that were infected with either *B. bovis*, *B. bigemina* or *A. centrale* were all negative in each genotype-specific assay. The performance of each assay was further assessed on 15 well-characterised blood samples from *T. orientalis*-positive animals and yielded comparable results to conventional PCR (Eamens et al., 2013c).

The Ikeda, Chitose and Buffeli qPCR assays were applied to DNA extracts from 10 naïve cattle that had been introduced onto a property with a recent history of clinical theileriosis. The 10 animals became infected with all three genotypes over the study period, with the Ikeda allele the first to be detected in the majority of animals (7/10 cows positive at Day 11 and the remaining three cows positive at Day 20). The Chitose allele was also detectable in Cow 8 at 11 days post-introduction, but was not detected in the other nine animals until Day 20. Buffeli allele was detected in nine animals at Day 20 and the remaining animal at Day 34. Together these data suggest that the Ikeda genotype may be either favoured during vector transmission, or out-competes the Chitose and Buffeli genotypes. Interestingly, in all animals the dominant genotype, as detected by qPCR, shifted over the course of the study, from the Ikeda genotype to the Chitose genotype, with the Ikeda genotype re-emerging as the dominant allele around Day 60 in most cases (Figure 2). Quantitative data for each genotype

revealed that the levels of Ikeda allele peaked 35-40 days post-introduction in the majority of animals (Figure 2). In most animals, the concentration of Chitose allele peaked only after the initial peak in the Ikeda genotype, from 40-55 days post-introduction. These peaks coincided with the peak in parasitaemia as determined by blood film, which in most animals was at Day 40 (Figure 2). The Buffeli genotype, while detected in all animals over the course of the study, was usually detected at concentrations an order of magnitude lower than the Ikeda and Chitose alleles. One exception was Cow 1, in which the Buffeli genotype reached a higher concentration than the Ikeda and Chitose genotypes. Despite this, the peak in parasite DNA in Cow 1 was 1-2 orders of magnitude lower than in the other nine animals. Furthermore, Cow 1 was the only animal that did not become anaemic during the course of the study. Eight of the remaining nine animals first became anaemic approximately eight days following the peak in concentration of the Ikeda genotype, and around the time when the Chitose genotype was peaking, the exception being Cow 10 which first became anaemic at Day 34, coinciding with the peak concentration of the Ikeda allele.

The Chitose subpopulation genotyping assay was applied to samples from the time course study where the Chitose genotype was at its peak. Of the ten samples, six were predominantly (>95%) Chitose A allele (Cows 2, 4, 5, 6, 7 and 8) and the remaining four samples were a mix of Chitose A and B. Of the samples containing a mix of Chitose A and B, Cows 9 and 10 contained a much higher proportion of Chitose A (75% and 86% respectively), while Cows 1 and 3 contained closer to an equimolar mix of the two alleles (43% and 52% Chitose A respectively). Quantitative data from the Chitose qPCR assay also demonstrated that Cows 1 and 3 had the lowest overall concentration of the Chitose allele when this genotype was at its peak.

4. Discussion

The aim of this study was to explore subpopulations and dynamics of *T. orientalis* MPSP genotypes in field-affected cattle. It has been well established that both clinical and subclinical *T. orientalis* infections frequently occur as a mixture of genotypes (Bogema et al., 2015; Eamens et al. 2013b, Kamau et al., 2011, Ota et al., 2009; Sivakumar et al., 2014), a phenomenon characteristic of apicomplexan parasites (Nkhoma et al. 2012; Katzer et al. 2006). While the Ikeda genotype of *T. orientalis* has been clearly associated with clinical outbreaks of disease, in Australian herds it has been demonstrated to co-occur with the Chitose genotype with high frequency (Eamens et al., 2013c) in the presence or absence of benign genotypes (Buffeli and Type 5) (Eamens et al., 2013c; Perera et al., 2013). In this study, naïve cattle introduced to a *Theileria*-endemic area rapidly became infected with a mix of MPSP genotypes (Ikeda, Chitose and Buffeli). It was not established whether these mixed infections arose from successive inoculations of distinct parasite populations from multiple tick bites, or transmission of multiple sporozoite genotypes from individual ticks. However, evidence from field studies of mosquito transmission of the malaria parasite, *Plasmodium falciparum* (Nkhoma et al. 2012), suggest that genetic recombination within the insect vector is sufficient to generate a genetically diverse population of sporozoites. Indeed, recombination within the vector is believed to be the major source of genetic diversity in *P. falciparum*, rather than "superinfection" from multiple mosquitoes. It has been demonstrated that subsequent sporozoite inoculations are in fact suppressed once blood-stage parasites reach a minimum density threshold (Portugal et al., 2011). Multiple MPSP genotypes have been detected within *T. orientalis* sporozoites harvested from *Haemaphysalis longicornis* ticks in Japan (Kubota et al., 1996), suggesting that mixed genotypes of this parasite can be transmitted in a single infective bite. *H. longicornis* is a competent vector for the transmission of the Ikeda and Chitose genotypes in Japan and while *H. longicornis* is present

in Australia, whether this species acts as a vector for the various *T. orientalis* genotypes is yet to be confirmed.

The observed temporal and magnitudinal dynamics of the *T. orientalis* genotype populations observed in this study could be explained by vector and/or immunological factors. The pathogenic Ikeda genotype was the first to be detected in the blood of all of ten cattle post-introduction to the affected property, with the levels of the Chitose and Buffeli genotypes peaking after the Ikeda genotype had reached its peak. This pattern was consistent in all 10 animals examined. Prior studies on *Theileria parva* demonstrated that different tick populations display distinct preferences for particular genotypes of the parasite (Katzer et al., 2006). Repeated early detection of the Ikeda genotype is consistent with transmission of a sporozoite population that is skewed towards this genotype, although host immunological factors could also account for this phenomenon (see later). Experimental transmission studies conducted in the 1980s suggested that populations of *H. longicornis* from Queensland were unable to transmit the Buffeli genotype, while *H. humerosa* and *H. bancrofti* were competent vectors (Stewart et al., 1987; Stewart et al., 1989). However, of these three potential vectors, only *H. longicornis* is known to be endemic to the mid-coast of NSW where the herd examined in this study was located and therefore is the most likely candidate vector. The relatively lower levels of Buffeli genotype observed in this study is consistent with the idea that *H.longicornis* is not a competent vector for transmitting Buffeli genotypes but further research is needed to assess the epidemiological role of *Haemaphysalis* sp. ticks in *T. orientalis* genotype transmission dynamics. Selection of the Ikeda and Chitose (especially Chitose A) genotypes within the tick vector could also explain the common co-occurrence of these genotypes reported here and elsewhere (Eamens et al., 2013b; Eamens et al., 2013c). Preferential selection of genotypes within the tick could also explain apparent differences in the geographical prevalence of the Chitose A versus Chitose B allele. The Chitose genotype

was found to be the most prevalent genotype of *T. orientalis* in Queensland herds during a recent survey (Eamens et al., 2013a); however, all of the Chitose-positive samples sourced from Queensland that were tested in this study predominantly harboured the Chitose B allele. In addition to the natural boundaries of potential vector species (Riek, 1982), local controls of cattle movements at the Queensland-NSW border, including mandatory drenching for ticks, may assist in creating genetically divergent intraspecies tick populations with disparate parasite genotype preferences.

The host immunological response likely played a key role in the epidemiology of theileriosis outbreaks in Australia and the genotype switching of *T. orientalis* observed in this study. While *T. orientalis* has been enzootic to parts of Australia for approximately 100 years, since 2006 there has been a large increase in the number of clinical cases attributed to this parasite (Eamens et al., 2013c; Izzo et al., 2010; Perera et al. 2013). This increase in clinical cases is attributed to the detection of a new genotype in Australia (Ikeda), while previous observations of *Theileria* in Australian cattle were presumed to be of the benign Buffeli genotype (Kamau et al. 2011). In *P. falciparum* infections, immunity to one allelic variant is not necessarily cross-protective for another (Jordan et al., 2011), therefore it is reasonable to assume that lack of a cross-protective response between *T. orientalis* genotypes in Australian cattle may have contributed to the rapid spread of clinical theileriosis since 2006. Additionally, lack of immune suppression of type Ikeda in cattle that had not been previously exposed to this genotype would allow out-competition of genotypes to which cattle had already been exposed. Allelic diversity within the Ikeda genotype (eg. at loci other than the MPSP) may explain the apparent re-emergence of this MPSP genotype in the majority of cattle towards the end of the sampling period. Further studies on *T. orientalis* populations using markers additional to the MPSP gene are warranted to fully elucidate the genetic diversity within this species and how these populations interact with the host immune system.

Prior research conducted in Japan using experimentally-infected splenectomised calves have suggested that the Ikeda and Chitose genotypes undergo temporal switching (Kubota et al., 1996; Matsuba et al., 1993). Temporal switching of these same genotypes was observed in the field-affected cattle monitored in this study, although switching occurred more rapidly than reported for the splenectomised animals. Kubota et al. (1996) reported that in splenectomised calves, the Ikeda genotype dominated at 38 days post-inoculation with and Ikeda-Chitose mix of sporozoites. The infection then switched to a dominant Chitose infection by Day 48 which persisted for the course of the study (120 days), only switching back to an Ikeda-dominant infection in some animals at 115 days post-inoculation. The more rapid switching observed in this study is likely related to the intact status of the cattle examined, compared to the splenectomised animals used in the prior studies, creating a greater immunological selection pressure for more rapid shifting. Indeed, prior studies on the malaria parasite, *Plasmodium falciparum* suggest that the spleen plays an important role in the sequestration of erythrocytes containing the parasite (Contamin et al., 2000), with different parasite variants inducing the expression of alternate antigens on the erythrocyte cell surface, thereby allowing the parasite to avoid splenic destruction (Borst et al., 1995). While the role of MHC in immune modulation of *T. orientalis* has not been confirmed, in *T. parva* and *T. annulata* infection, $CD8^+$ T cell responses are directed against class I MHC-bound antigenic peptides and display a clear dominance hierarchy (MacHugh et al., 2009; MacHugh et al. 2011). T cell responses are restricted further by the specific MHC haplotypes of host cattle and in *T. parva* infection, the majority of these responses are focussed on a single antigenic epitope (MacDonald et al. 2010). A similarly restricted immune response to *T. orientalis* infection could explain the relative pathogenicity of the different genotypes as well as the relative susceptibility of individual animals to pathogenic forms of the parasite. Although stress (transport, pregnancy, lactation) has been widely reported as a disease trigger

(Eamens et al., 2013c; Izzo et al., 2010), the inter- and intra-breed susceptibility of cattle to *T. orientalis* infection has not been extensively investigated. There is however, some suggestion that there may be differential susceptibility of *Bos taurus* versus *Bos indicus* breeds (Yang et al., 2014) and that Japanese black cattle (Wagyu) may be less susceptible to disease (Terada et al., 1995). The animals tested in our temporal study were all of a single breed (Ayrshire) and the patterns of genotype switching and disease progression were remarkably similar amongst the majority of animals. One exception was Cow 1 which contained peak parasite levels 1-2 orders of magnitude lower than its cohorts, did not become anaemic over the course of the study, and ultimately displayed infection dominated by the Buffeli genotype. These differences in the progression of infection may well be related to the nature of the immune response in Cow 1 resulting from the interplay between host and parasite genotype.

While the Ikeda genotype of *T. orientalis* has been clearly linked to clinical disease, the pathogenicity of the Chitose genotype is less clear. While this genotype frequently co-occurs with type Ikeda, it has only rarely been suggested to be the sole cause of disease (McFadden et al., 2011). Futhermore, McFadden et al., used the 18S rRNA and cytochrome oxidase III genes, rather than the MPSP gene to characterise their isolate, therefore the MPSP genotype of this isolate remains unconfirmed. Indeed, our recent research shows the Chitose genotype is only associated with high parasite loads when occurring in combination with type Ikeda (Bogema et al., 2015). Here, we identified a statistically higher association between clinical disease and the presence of the Chitose A compared to the Chitose B genotype; however it was noted that the Chitose A genotype almost always occurs with type Ikeda (~95% of cases examined); therefore there is little evidence to suggest that Chitose A is a direct cause of clinical disease. While qPCR and allele frequency analysis revealed that the Chitose A genotype contributed significantly to the overall parasite burden in some animals, particularly during the acute phase of the disease where animals became anaemic, an analysis of clinical

parameters in sole Ikeda versus mixed Ikeda-Chitose infections is required to determine whether this genotype exacerbates disease. A recent introduction of the Ikeda genotype to Australia, co-inciding with the increase in disease outbreaks, is believed to have occurred, with a phylogenetic relationship between Australian and Japanese Ikeda isolates proposed (Perera et al. 2015). The phylogenetic analysis of the Chitose sequences presented here also supports a Japanese origin for Chitose A but not for the Chitose B genotype. Combined with the strong association observed between the Chitose A and Ikeda genotypes, we postulate that these genotypes were likely introduced to Australia at the same time; however further studies on less highly conserved molecular markers are warranted to investigate this further. In contrast, a Chitose type B MPSP sequence was detected in a Japanese study (Kim et al. 1998) from animal imported from Australia as early as 1996, suggesting that Chitose B may have been present in Australia for some time prior to the disease outbreaks.

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Figure legends:

Figure 1. Phylogenetic tree summarising results from neighbour-joining, Maximum Likelihood and Maximum Parsimony analyses. Bootstrap values were calculated using each method. The tree depicted was generated using the Maximum Likelihood method and the bootstrap values are indicated by closed circles (>50% support in all three analyses) and open circles (<50% support in one or more analysis). The Chitose A and Chitose B phylogenetic were well-supported in all three analyses. Sequences derived from this study are shown in bold.

Figure 2: Temporal population dynamics of three *T. orientalis* subtypes in 10 field-infected cattle. For each cow, Top: PCV (dotted line) and parasitaemia as determined by blood film analysis (solid line). Bottom: Allele concentrations determined by qPCR targeting the Ikeda (dashed line), Chitose (solid line) and Buffeli (dotted line) genotypes. Note that y-axes are not equivalent for each cow.

Table 1: Oligonucleotides used in this study.

Oligo	**Sequence (5′-3′)**	**Target**	**Working concentration**	**Reference**
Ikeda quantitative assay				
MPSP-I-F	ATTGGTAGACGGAAAATGGAAGAAGG	Ikeda MPSP gene	900 nM	This study
MPSP-I-R	GAGACTCAATGCGCCTAGAGATAATAGA	Ikeda MPSP gene	900 nM	This study
Pr-I	VIC-CATGAACAGTGCTTGGC -MGB-NFQ	Ikeda MPSP gene	250 nM	Bogema et al, 2015)
Chitose quantitative assay				
MPSP-C-F	CCGGTGATGAGAGATTCAAGGAAGTA	Chitose MPSP gene	900 nM	This study
MPSP-C-R	GACTCAATGCGCCTAGARATAGTAGG	Chitose MPSP gene	900 nM	This study
Pr-Ca	NED-TCCTCAGCGCTGTTCT-MGB-NFQ	Chitose A MPSP gene	100 nM	Bogema et al, 2015)
Pr-Cb	NED-TCCTCGGCGCTGTTCT-MGB-NFQ	Chitose B MPSP gene	150 nM	Bogema et al, 2015)
Buffeli quantitative assay				
MPSP-B-F	AAGTATACGTAGGTACCGATGATAAGAAAGTA	Buffeli MPSP gene	900 nM	This study
MPSP-B-R	GAGACTCAATGCGCCTAGAGATAGA	Buffeli MPSP gene	900 nM	This study
Pr-B	FAM-AGCGCTTTCCTCATCG-MGB-NFQ	Buffeli MPSP gene	250 nM	This study
Chitose subpopulation assay				
MPSP-C-F	TCGACAAGTTCTCACCAC	Chitose MPSP gene	900 nM	This study
MPSP-C-R	CATGAACAGTGCTTGGC	Chitose MPSP gene	900 nM	This study
Pr-Ca (VIC)	VIC-TCCTCAGCGCTGTTCT-MGB-NFQ	Chitose A MPSP gene	150 nM	This study
Pr-Cb	NED-TCCTCGGCGCTGTTCT-MGB-NFQ	Chitose B MPSP gene	150 nM	Bogema et al, 2015)
Primers used for generation and sequencing of standard plasmids				
TsI	CACCATCGTCTGCTACCGCCGC	Ikeda MPSP gene (cPCR forward and sequencing primer)	400 nM	(Zakimi et al., 2006)
TsC	CACCTTCCTCATCGTCTCTGCAACT	Chitose (cPCR forward and sequencing primer)	400 nM	(Zakimi et al., 2006)
TsB	CACCTTCCTCATCGTCTCTGCAACT	Buffeli (cPCR forward and sequencing primer)	400 nM	(Zakimi et al., 2006)
TsR	CACCTGCTCTGCAACCGCAGAG	*T. orientalis* (cPCR reverse and sequencing primer)	400 nM	(Zakimi et al., 2006)
Chb mutF	CTATGTGAGACTCAATGCGCCTA	Chitose plasmid mutagenesis forward primer	400 nM	Bogema et al, 2015)
Chb mutR	GATGAGAACAGCGCCGAGGACGGCAAGTG	Chitose plasmid mutagenesis reverse primer	400 nM	Bogema et al, 2015)
T7F	CACTTGCCGTCCTCGGCGCTGTTCTCATC	pET100/GW/D-TOPO vector forward primer	830 nM	Bogema et al, 2015)
T7R	TAATACGACTCACTATAGGG	pET100/GW/D-TOPO	830 nM	Bogema et

	vector reverse primer	al, 2015)

Table 2: Relative occurrence of Chitose A and B alleles in Chitose PCR positive samples and their association with clinical disease cases and the Ikeda genotype.

Allele frequency	% associated with clinical disease	% co-occurrence with Ikeda genotype
>95% Chitose A (n = 56)	100% (56)	98% (55)
Mix Chitose A/B (n = 52)	67% (35)	90.4% (47)
>95% Chitose B (n = 29)	27.6% (8)	44.8% (13)

Table 3: Relative occurrence of the Chitose A and B alleles in Chitose PCR positive samples by geographic origin

Allele frequency	Geographic origin by State			
	NSW	QLD	VIC	SA
>95% Chitose A (n = 56)	56	0	0	0
Mix Chitose A/B (n = 52)	45	0	3	2
>95% Chitose B (n = 29)	11	16	4	0
Total	112	16	7	2

Table 4: Analytical sensitivity (limit of detection, LOD) of the Ikeda- Chitose- and Buffeli-specific assays, 95% confidence limits are shown in brackets.

qPCR Parameter	Ikeda	Chitose	Buffeli
R^2	0.997	0.999	0.994
Efficiency (%)	91.2 (87.9 - 94.9)	95.1 (92.9 - 97.4)	90.7 (86.4 - 95.3)
LOD ($GC.\mu L^{-1}$)	8.4 (3.1 - 23)	35 (14 - 88)	19 (8.5 - 44)

Figure 1

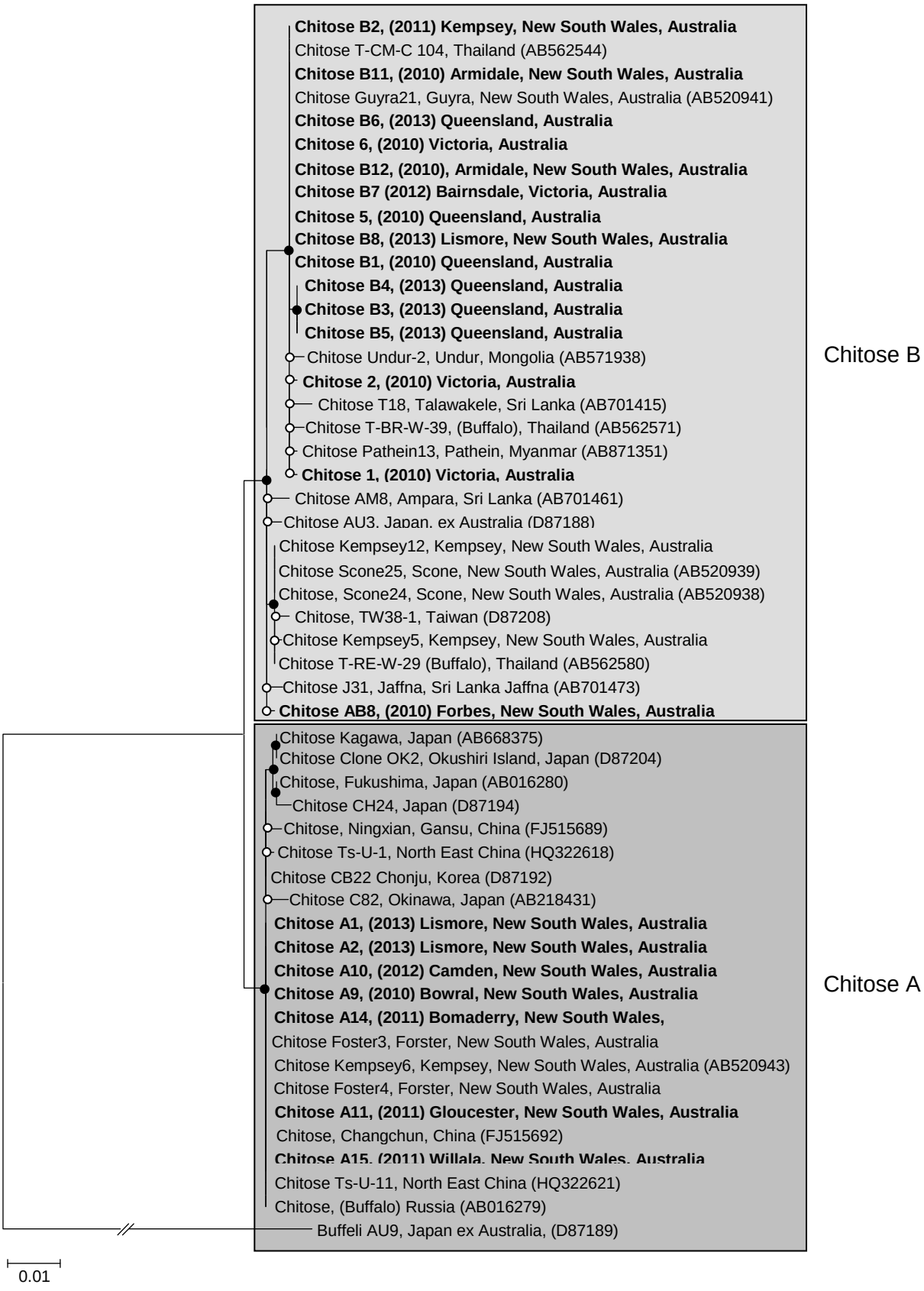
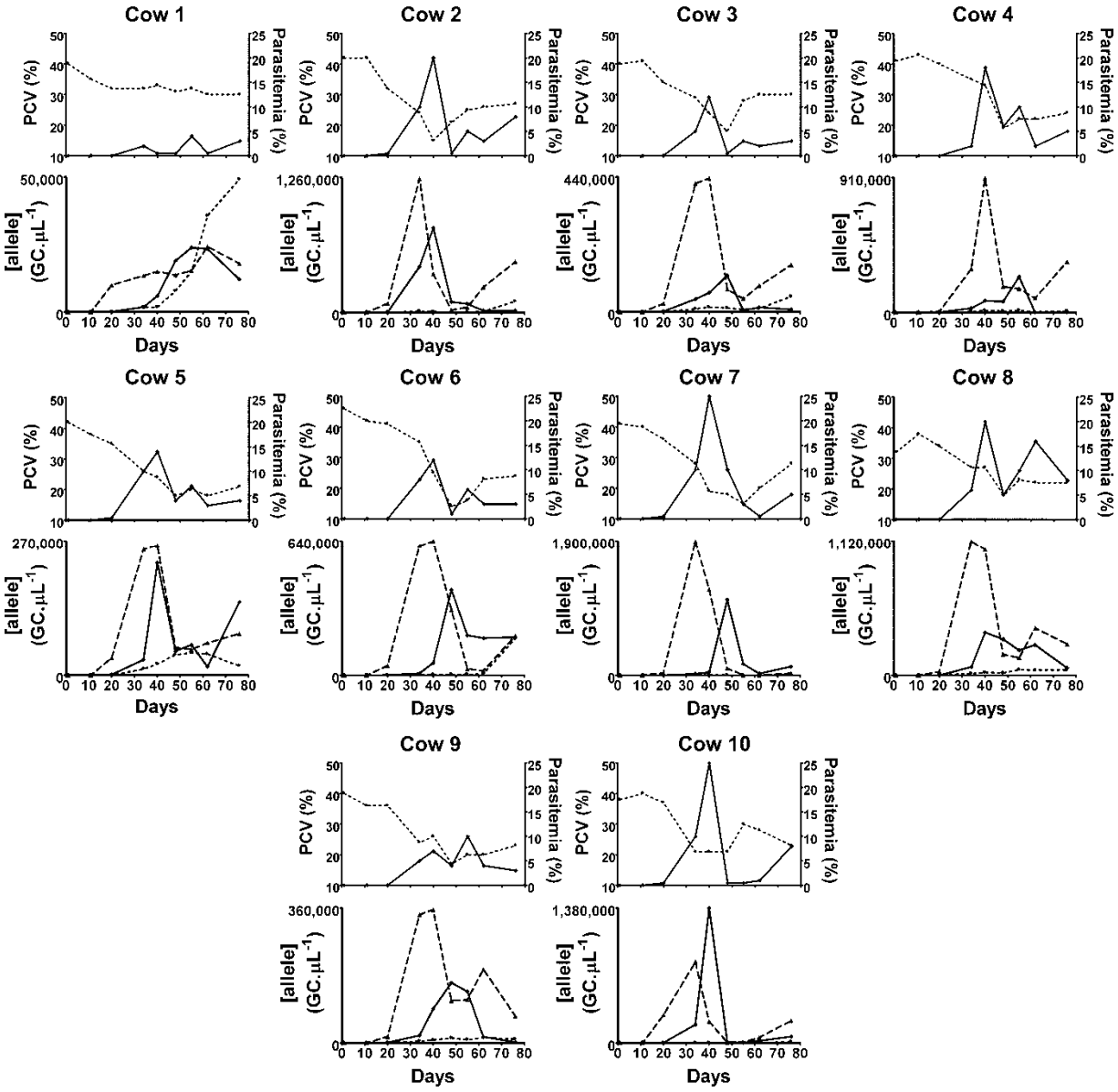

Chitose B2, (2011) Kempsey, New South Wales, Australia
Chitose T-CM-C 104, Thailand (AB562544)
Chitose B11, (2010) Armidale, New South Wales, Australia
Chitose Guyra21, Guyra, New South Wales, Australia (AB520941)
Chitose B6, (2013) Queensland, Australia
Chitose 6, (2010) Victoria, Australia
Chitose B12, (2010), Armidale, New South Wales, Australia
Chitose B7 (2012) Bairnsdale, Victoria, Australia
Chitose 5, (2010) Queensland, Australia
Chitose B8, (2013) Lismore, New South Wales, Australia
Chitose B1, (2010) Queensland, Australia
Chitose B4, (2013) Queensland, Australia
Chitose B3, (2013) Queensland, Australia
Chitose B5, (2013) Queensland, Australia
Chitose Undur-2, Undur, Mongolia (AB571938)
Chitose 2, (2010) Victoria, Australia
Chitose T18, Talawakele, Sri Lanka (AB701415)
Chitose T-BR-W-39, (Buffalo), Thailand (AB562571)
Chitose Pathein13, Pathein, Myanmar (AB871351)
Chitose 1, (2010) Victoria, Australia
Chitose AM8, Ampara, Sri Lanka (AB701461)
Chitose AU3, Japan, ex Australia (D87188)
Chitose Kempsey12, Kempsey, New South Wales, Australia
Chitose Scone25, Scone, New South Wales, Australia (AB520939)
Chitose, Scone24, Scone, New South Wales, Australia (AB520938)
Chitose, TW38-1, Taiwan (D87208)
Chitose Kempsey5, Kempsey, New South Wales, Australia
Chitose T-RE-W-29 (Buffalo), Thailand (AB562580)
Chitose J31, Jaffna, Sri Lanka Jaffna (AB701473)
Chitose AB8, (2010) Forbes, New South Wales, Australia
Chitose B
Chitose Kagawa, Japan (AB668375)
Chitose Clone OK2, Okushiri Island, Japan (D87204)
Chitose, Fukushima, Japan (AB016280)
Chitose CH24, Japan (D87194)
Chitose, Ningxian, Gansu, China (FJ515689)
Chitose Ts-U-1, North East China (HQ322618)
Chitose CB22 Chonju, Korea (D87192)
Chitose C82, Okinawa, Japan (AB218431)
Chitose A1, (2013) Lismore, New South Wales, Australia
Chitose A2, (2013) Lismore, New South Wales, Australia
Chitose A10, (2012) Camden, New South Wales, Australia
Chitose A9, (2010) Bowral, New South Wales, Australia
Chitose A14, (2011) Bomaderry, New South Wales,
Chitose Foster3, Forster, New South Wales, Australia
Chitose Kempsey6, Kempsey, New South Wales, Australia (AB520943)
Chitose Foster4, Forster, New South Wales, Australia
Chitose A11, (2011) Gloucester, New South Wales, Australia
Chitose, Changchun, China (FJ515692)
Chitose A15, (2011) Willala, New South Wales, Australia
Chitose Ts-U-11, North East China (HQ322621)
Chitose, (Buffalo) Russia (AB016279)
Buffeli AU9, Japan ex Australia, (D87189)
Chitose A
0.01

Figure 2.


Cow 1
Cow 2
Cow 3
Cow 4
Cow 5
Cow 6
Cow 7
Cow 8
Cow 9
Cow 10
PCV (%)
Parasitemia (%)
[allele] (GC.µL-1)
Days