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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,c; 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.,

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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). 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. Phillip 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 703 bp of sequence analysed. All sequences have been submitted to GenBank (Accession numbers KP313255–KP313276). 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 (i.e. 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)