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Ancient diversity and geographical sub-structuring in African buffalo *Theileria parva* populations revealed through metagenetic analysis of antigen-encoding loci ☆

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ABSTRACT

An infection and treatment protocol involving infection with a mixture of three parasite isolates and simultaneous treatment with oxytetracycline is currently used to vaccinate cattle against *Theileria parva*. While vaccination results in high levels of protection in some regions, little or no protection is observed in areas where animals are challenged predominantly by parasites of buffalo origin. A previous study involving sequencing of two antigen-encoding genes from a series of parasite isolates indicated that this is associated with greater antigenic diversity in buffalo-derived *T. parva*. The current study set out to extend these analyses by applying high-throughput sequencing to ex vivo samples from naturally infected buffalo to determine the extent of diversity in a set of antigen-encoding genes. Samples from two populations of buffalo, one in Kenya and the other in South Africa, were examined to investigate the effect of geographical distance on the nature of sequence diversity. The results revealed a number of significant findings. First, there was a variable degree of nucleotide sequence diversity in all gene segments examined, with the percentage of polymorphic nucleotides ranging from 10% to 69%. Second, large numbers of allelic variants of each gene were found in individual animals, indicating multiple infection events. Third, despite the observed diversity in nucleotide sequences, several of the gene products had highly conserved amino acid sequences, and thus represent potential candidates for vaccine development. Fourth, although compelling evidence for population differentiation between the Kenyan and South African *T. parva* parasites was identified, analysis of molecular variance for each gene revealed that the majority of the underlying nucleotide sequence polymorphism was common to both areas, indicating that much of this aspect of genetic variation in the parasite population arose prior to geographic separation.

1. Introduction

Antigenic heterogeneity is a key feature of a number of important protozoan pathogens of animals and humans, including *Plasmodium* spp. (Kyes et al., 2001), *Eimeria tenella* (Blake et al., 2015) and *Theileria parva* (Morrison et al., 2015). In the latter two species, live parasites are used for vaccination and inclusion of more than one parasite genotype in the vaccines is required to provide protection in the field. However, until recently, the antigenic basis of the immunological heterogeneity of these parasites has been poorly understood.

The apicomplexan parasite *T. parva*, transmitted by the three-host tick *Rhipicephalus appendiculatus*, infects cattle and African buffalo (*Syncerus caffer*). It causes an acute, often fatal lymphoproliferative disease in cattle, known as East Coast fever (ECF), which is a major constraint to livestock production throughout a large

☆ DNA sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession numbers SRX2060341 to SRX2060423. Sample metadata was deposited in the NCBI BioSample database under accession numbers 5712932–5712932.

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part of eastern and southern Africa (Irvin and Morrison, 1987). The parasite infects lymphocytes, in which it develops to the multinucleate schizont stage, which induces activation and proliferation of infected cells (Dobbelaere and Rottenberg, 2003). By associating with the host cell spindle during mitosis, the parasite is able to divide at the same time as the host cell, resulting in maintenance of infection in the daughter cells (von Schubert et al., 2010). In cattle, this process leads to progressive expansion of the parasitised cell population and severe disease (Morrison et al., 1981). The subsequent intra-erythrocytic piroplasm stage causes little or no pathology, but is required for onward transmission by ticks. Animals that manage to recover from the acute phase of disease develop low level carrier infections which can persist for many months or years and are an important source of parasites for tick transmission (Kariuki et al., 1995; Skilton et al., 2002).

Although *T. parva* is able to infect and transform buffalo lymphocytes in the same manner as cattle cells, infections in buffalo are not associated with overt clinical signs. Most buffalo residing in ECF-endemic areas are infected with *T. parva* (Young et al., 1978) and hence they represent a reservoir for infection of cattle. Cattle that acquire infection from ticks that have fed on buffalo develop severe disease, but these parasites differentiate poorly to the tick-infective piroplasm stage in cattle and therefore are not transmitted or are transmitted at low efficiency by ticks to other cattle (Neitz, 1957; Schreuder et al., 1977; Grootenhuis et al., 1987; Mbizeni et al., 2013). Available data suggest that, as a consequence of this barrier, populations of *T. parva* found in buffalo differ genotypically from those maintained in cattle (Oura et al., 2011; Pelle et al., 2011). In South Africa, *T. parva* is currently confined to buffalo, cattle-maintained *T. parva* having been eradicated in the first half of the last century and transmission from buffalo to cattle controlled by strict separation of infected buffalo from cattle (Norval et al., 1992).

In the 1970s a method of vaccinating cattle against *T. parva* was developed, involving infection with *T. parva* sporozoites and simultaneous treatment with long-acting tetracycline (reviewed in Morrison and McKeever, 2006). Although immunisation of cattle with one parasite isolate resulted in solid long-lasting immunity to challenge with the same isolate, a proportion of immunised animals remained susceptible to challenge with other isolates (Radley et al., 1975a). Following a series of immunisation and challenge experiments, a combination of three parasite isolates was identified which generated immunity against experimental challenge with a range of *T. parva* isolates of cattle origin (Radley et al., 1975b) and against field challenge (Uilenberg et al., 1976). This mixture of parasites, known as the Muguga cocktail, is currently used to vaccinate against the disease in eastern Africa (Di Giulio et al., 2009).

Studies of immune cattle have indicated that parasite-specific $CD8^+$ T cell responses play a key role in immunity and have shown that lack of cross-protection between parasite isolates is reflected by strain specificity of the $CD8^+$ T cell responses (McKeever et al., 1994; Taracha et al., 1995a,b). Although the Muguga cocktail vaccine has been used successfully in some regions, there is evidence that it does not protect all animals against infections acquired by transmission from buffalo (Radley et al., 1979). Thus, two recent studies found that vaccinated cattle introduced into areas grazed solely or predominantly by buffalo showed no or low levels of protection against disease (Bishop et al., 2015; Sitt et al., 2015). These findings imply that *T. parva* parasite populations in buffalo harbour a greater antigenic diversity than those in cattle. Evidence to support this contention was provided by a study of the sequences of two genes (Tp1 and Tp2) which encode proteins recognised by $CD8^+$ T cells from immune cattle, in infected cell lines isolated primarily from eastern Africa (Pelle et al., 2011). Over 30 allelic variants of each antigen were identified, and a large majority of these were found in isolates obtained from buffalo or from cattle that had grazed alongside buffalo, whereas only a small subset of the variants was detected in the isolates obtained from cattle grazed in the absence of buffalo (Pelle et al., 2011).

Given the evidence of genetic diversity in buffalo populations of *T. parva*, the current study set out to extend these analyses by applying a metagenetic, high-throughput sequencing approach to determine the extent of polymorphism in a panel of antigen-encoding genes. Samples from two geographically distant populations of buffalo, one in Kenya and the other in South Africa, were examined in order to investigate the influence of geographic separation on parasite genetic diversity. The results demonstrate extensive allelic diversity in all genes examined, both at the individual animal and population levels of the buffalo, but also reveal that some of the parasite antigen genes show a high level of conservation at the amino acid level. Comparison between the Kenyan and South African *T. parva* sequences indicates that much of the underlying genetic variation is found in both buffalo populations and is therefore likely to have been present in a common ancestor prior to geographical separation.

2. Materials and methods

2.1. Buffalo DNA

Samples of genomic DNA were obtained from single blood samples from eight African buffalo on the Ol Pejeta Conservancy in the Laikipia district of Kenya (Sitt et al., 2015) and from six buffalo in the Kruger National Park, South Africa. Ethical approval for the project was provided by the International Livestock Research Institute (ILRI), Kenya through its Institutional Animal Care and Use Committee (IACUC Approval 2011–11). Permission for the collection of samples from Ol Pejeta Conservancy was provided by the Kenya Wildlife Service, Kenya (KWS/BRM/5001) and from culled buffalo in the Kruger National Park by the South African National Parks, South Africa. DNA was extracted from the Kenyan buffalo samples using a DNeasy Blood and Tissue Kit (Qiagen, USA). The South African samples were provided by Dr. Nicholas Juleff, Pirbright Institute, UK. Further information on the origin of the buffalo, their sex and estimated age can be found in the National Center for Biotechnology Information, USA (NCBI) BioSample database under accession numbers 5712932–5712932.

2.2. Sequencing of 18S rRNA subunit

The presence of different species of *Theileria* and *Babesia* in the blood samples was investigated by sequencing a 375 bp segment of the 18S rRNA gene using Roche 454 amplicon sequencing technology as previously described (Bishop et al., 2015).

2.3. Sequencing T. parva antigen-encoding genes

A high-throughput multi-locus sequence typing system involving Roche 454 sequencing of PCR amplicons of selected *T. parva* genes was employed to identify allelic variation in the target genes. Amplicons of segments (292–492 bp) of six genes known to encode antigens capable of recognition by bovine $CD8^+$ T cells (Tp1, Tp2, Tp4, Tp5, Tp6 and Tp10) (Graham et al., 2006; Hemmink et al., 2016) were generated as previously described (Hemmink et al., 2016). Regions containing known $CD8^+$ T cell epitopes were selected. The genomic references of these genes, the gene-specific primer sequences and the PCR conditions used are reported elsewhere (Hemmink et al., 2016). For highly polymorphic loci such as Tp2, degenerate primers were selected to facilitate amplification from a diverse range of allelic sequences. In