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Original article

Assessment of sequence variability in a *p23* gene region within and among three genotypes of the *Theileria orientalis* complex from south-eastern Australia

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ABSTRACT

Oriental theileriosis is a tick-borne, protozoan disease of cattle caused by one or more genotypes of *Theileria orientalis* complex. In this study, we assessed sequence variability in a region of the 23 kDa piroplasm membrane protein (*p23*) gene within and among three *T. orientalis* genotypes (designated *buffeli*, *chitose* and *ikeda*) in south-eastern Australia. Genomic DNA ($n = 100$) was extracted from blood of infected cattle from various locations endemic for oriental theileriosis and tested by polymerase chain reaction (PCR)-coupled mutation scanning (single-strand conformation polymorphism (SSCP)) and targeted sequencing analysis. Eight distinct sequences represented all DNA samples, and three genotypes were found: *buffeli* ($n = 3$), *chitose* (3) and *ikeda* (2). Nucleotide pairwise comparisons among these eight sequences revealed considerably higher variability among the genotypes (6.6–11.7%) than within them (0–1.9%), indicating that the *p23* gene region allows the accurate identification of *T. orientalis* genotypes. In the future, we will combine this gene with other molecular markers to study the genetic structure of *T. orientalis* populations in Australasia, which will pave the way to establish a highly sensitive and specific PCR-based assay for genotypic diagnosis of infection and for assessing levels of parasitaemia in cattle.

Introduction

Oriental theileriosis is a malaria-like disease of bovines, caused by protozoan parasites within the *Theileria orientalis* complex. Members of this complex are transmitted by ixodid ticks (primarily *Haemaphysalis* spp.), and invade the host and replicate in the blood stream within erythrocytes, causing anaemia and associated complications, such as high fever, jaundice, lethargy, weakness, abortion and/or mortality (Izzo et al., 2010; Aparna et al., 2011; McFadden et al., 2011). This form of theileriosis occurs mainly in the Asia-Pacific region (Izzo et al., 2010; Aparna et al., 2011; Islam et al., 2011; McFadden et al., 2011) and, recently, there has been a number of serious outbreaks in Australasia (Izzo et al., 2010; Islam et al., 2011; McFadden et al., 2011).

The development of practical molecular tools for the accurate diagnosis of *T. orientalis* infections and/or analysis of genetic variation is crucial for epidemiological investigations (e.g., to determine prevalence and transmission patterns), inferring which genotypes of *T. orientalis* link to clinical disease, monitoring the spread of disease and tracking outbreaks. Central to establishing such tools is the selection of suitable genetic markers for the detection of particular genotypes of *T. orientalis*, as it is recognised that only some genotypes are virulent (Kamau et al., 2011a; Perera et al., 2013). To date, the major piroplasm surface protein (*mpsp*), small subunit (*SSU*) of nuclear ribosomal RNA and 23-kDa piroplasm membrane protein (*p23*) genes as well as the first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2) have been used to study the genetic composition of the *T. orientalis* complex (Sako et al., 1999; Gubbels et al., 2000; Aktas et al., 2006; Ota et al., 2009; Kamau et al., 2011b; Yokoyama et al., 2011). The most widely applied marker has been *mpsp* (Jeong et al., 2010; Altangerel et al., 2011; Islam et al., 2011; Kamau et al., 2011a; Khukhuu et al., 2011; Yokoyama et al., 2011, 2012; Cufos et al., 2012; Eamens et al., 2013; Perera et al., 2013, 2014; Sivakumar et al., 2013). Employing this marker, at least 11 genotypes of *T. orientalis* (i.e., *chitose* or *type* 1; *ikeda* or *type* 2; *buffeli* or *type* 3; *types* 4–8; N-1; N-2 and N-3) have been defined (reviewed by Sivakumar et al., 2014).

Recently, we identified four genotypes (*buffeli*, *chitose*, *ikeda* and *type* 5) in south-eastern Australia using PCR-based mutation scanning (SSCP)-targeted sequencing (Perera et al., 2013), two of which (i.e., *ikeda* and *chitose*) are considered as most virulent in cattle

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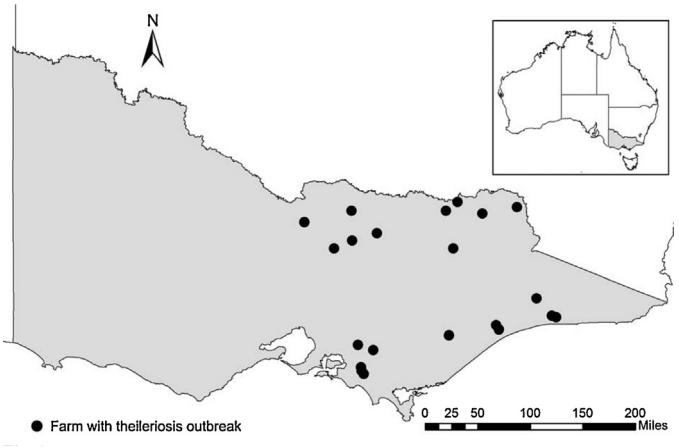

Fig. 1. Map showing geographical locations of farms ($n = 11$) from which cattle blood ($n = 100$) was collected in the state of Victoria, Australia (cf. Perera et al., 2013, 2014). Each dot on the map shows one farm that was affected by oriental theileriosis. Inset shows an Australian map with Victoria shaded in grey.

(Shimizu et al., 1992; Kamau et al., 2011a; Perera et al., 2013). In spite of this information, very little is known about the extent of sequence diversity in other genes within and among recognised genotypes of the *T. orientalis* complex. Assessing such diversity would provide a better insight into the genetic composition of *T. orientalis* and might also allow the definition of independent diagnostic markers for application in advanced PCR assays. In the present study, we assessed the magnitudes of sequence variability in part of the *p23* gene region within and among *T. orientalis* genotypes classified previously based on their *mpsp* sequences.

Materials and methods

Genomic DNA samples

Genomic DNA from 100 individual blood samples from cattle (*Bos taurus*) were available from previous studies (Perera et al., 2013, 2014) from 18 farms in endemic regions in Victoria, Australia (Fig. 1). Each DNA sample had been previously shown to contain only one of three genotypes of *T. orientalis* using single-strand conformation polymorphism (SSCP)-targeted sequencing analysis (Cufos et al., 2012) of part of the *mpsp* gene. In total, 10, 30 and 60 samples were shown to contain genotypes *buffeli*, *chitose* and *ikeda*, respectively.

PCR, agarose gel electrophoresis and SSCP-based sequencing

Oligonucleotide primers p23F1 (forward; 5′-ATATGGAAGGCTACAAGGCC-3′) and p23R1 (reverse; 5′-CAAGAGAGGCAACAACAACG-3′) were designed by aligning all published *p23* gene sequences (GenBank accession numbers AB021223, AB491348–AB491354, AB469176–AB469181, D84446 and D84447; Sako et al., 1999; Ota et al., 2009; Yokoyama et al., 2011) and used to specifically amplify a fragment (469 bp) of this gene by PCR from genotypes *buffeli*, *chitose* and *ikeda* of *T. orientalis*. The reagents and PCR conditions were optimised in a series of experiments; the final PCR was conducted in a 50 μl volume containing 10 mM Tris–HCl (pH 8.4), 50 mM KCl (Promega, USA), 3.5 mM $MgCl_2$, deoxynucleotide triphosphates (dNTPs; 200 μM each), primers (35 pmol each; GeneWorks, Australia) and 1 U Go*Taq* polymerase (Promega) using the following protocol: 5 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 64 °C and 30 s at 72 °C, followed by a final extension of 5 min at 72 °C. Following PCR, an aliquot (5 μl) of each amplicon was examined on 1.5% w/v agarose gels and photographed using GelDoc system (Bio-Rad, Hercules, CA, USA). Subsequently, SSCP analysis of amplicons ($n = 100$) was performed as described previously (Cufos et al., 2012; Perera et al., 2013); 1–3 amplicons per SSCP profile were selected, treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., USA) (Werle et al., 1994) and subjected to direct bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, USA) using the same primers individually as employed in PCR. The quality of sequences was assessed using the programme Geneious Pro 2.0.10 (Larkin et al., 2007).

Data and phylogenetic analyses

Nucleotide sequences of the *p23* gene fragment determined were compared with those of homologous reference sequences for genotypes *buffeli*, *chitose* and *ikeda* of *T. orientalis* in current databases (GenBank accession numbers AB021223, AB469178, AB491348 and AB491349 (for *buffeli*), AB469177, AB469179, AB491350, AB491351 and D84446 (for *chitose*), and AB469176, AB469180, AB469181, AB491352–AB491354 and D84447 (for *ikeda*)); sequence identities (in %) were calculated by pairwise comparison using the programme BioEdit (V7.2.5). All sequences obtained for the *p23* region were aligned over 469 nucleotide positions with homologous reference sequences of *T. orientalis* (GenBank accession nos. above; Sako et al., 1999; Ota et al., 2009; Yokoyama et al., 2011) and *Theileria annulata* (outgroup). The phylogenetic analysis of sequence data was conducted using Bayesian inference (BI), employing the Monte Carlo Markov Chain (MCMC) method of MrBayes 3.1.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) as described previously (Cufos et al., 2012; Perera et al., 2013). Briefly, the likelihood parameters were set according to the Akaike information criteria (AIC) in jModeltest v2.1.5 (Darriba et al., 2012). Four simultaneous tree-building chains were used to calculate posterior probabilities (pp) for 2,000,000 generations, saving every 100th tree produced. Based on the final 75% of trees generated, a consensus tree was constructed. In addition, an independent analysis was carried out using the Neighbour joining (NJ) method (Swofford, 1999) in the programme MEGA 6.06 (Tamura et al., 2013). Distance was estimated employing the general time-reversible model of evolution, and nodes were tested for robustness with 10,000 bootstrap replicates (Felsenstein, 1985).

Results and discussion

First, we amplified the *p23* region from all 100 genomic DNA samples and conducted an SSCP analysis to assess genetic variation among and within amplicons. The analysis of the amplicons displayed eight profiles (P1–P8) (see Fig. 2). Amplicons representing the unique SSCP profiles were subjected to direct sequencing, and eight distinct sequences were obtained. These eight sequences were deposited in the GenBank database under accession numbers KM504986–KM504993. Three of these sequences (KM504986–KM504988) represented SSCP profiles P1–P3, respectively, and genotype *buffeli*; three sequences (KM504989–KM504991) represented SSCP profiles P4–P6, respectively, and genotype *chitose*; and two sequences (KM504992–KM504993) represented profiles P7 and P8, respectively, and genotype *ikeda*.

Subsequently, we studied sequence variation in the *p23* region within each of the three genotypes (*buffeli*, *chitose* and *ikeda*) and differences among them. Within individual genotypes, nucleotide variability ranged from 0 to 1.9% (Tables 1–3; supplementary Figs. 1–3). Among these three genotypes, nucleotide variability ranged from 6.6% to 11.7% (data not shown). We then compared