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Molecular characterization of *Theileria orientalis* from cattle in Ethiopia

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ABSTRACT

This study reports the first molecular characterization of *Theileria orientalis* in local breeds of cattle in Ethiopia. A conventional PCR utilizing major piroplasm surface protein (*MPSP*) gene and an established multiplexed tandem PCR (MT-PCR) were used to characterize *T. orientalis* and to assess the infection intensity, respectively. Of 232 blood samples tested, *T. orientalis* DNA was detected in only 2.2% of samples using conventional PCR; two genotypes *buffeli* (1.3%; 3/232) and *type* 5 (0.9%; 2/232) of *T. orientalis* were detected. Phylogenetic analysis revealed that the *buffeli MPSP* sequences from Ethiopia were closely related to those reported from Kenya, Sri Lanka and Myanmar, and *type* 5 sequences from Ethiopia grouped with those from Korea, Japan, Vietnam and Thailand. A higher number of samples (3.9%; 9/232) were test-positive by MT-PCR and four genotypes (*buffeli*, *chitose*, *ikeda* and *type* 5) of *T. orientalis* were detected. The average intensity of infections with genotypes *buffeli* (DNA copy numbers 11,056) and *type* 5 (7508) were significantly higher ($P < 0.0001$) than the pathogenic genotype *ikeda* (61 DNA copies). This first insight into *T. orientalis* from cattle in Ethiopia using *MPSP* gene provides a basis for future studies of *T. orientalis* in various agroclimatic zones and of the impact of oriental theilerosis on cattle in this and other countries of Africa.

1. Introduction

Theileriosis is caused by tick-borne, haemoprotozoan parasites of the genus *Theileria* spp. and is one of the most economically important diseases of bovines, particularly in tropical and sub-tropical regions of the world (Mehlhorn and Schein, 1984; Uilenberg, 1995; Ahmed et al., 2002; Makala et al., 2003; Aktas et al., 2006). The most important *Theileria* spp. infecting bovines are *T. parva* and *T. annulata*, which cause East Coast fever and tropical theileriosis, respectively (Salih et al., 2007; Yusufmia et al., 2010), whereas other taxa, such as *T. mutans*, *T. velifera* and those of the *T. orientalis* complex are believed to cause milder and/or non-pathogenic theileriosis (Uilenberg et al., 1977; Salih et al., 2007; Tomassone et al., 2012). However, recently, *T. orientalis* complex, transmitted mainly by *Haemaphysalis* spp. caused significant morbidity, economic losses and/or mortality in cattle in the Asia-Pacific region (Izzo et al., 2010; Aparna et al., 2011; Kamau et al., 2011; McFadden et al., 2011; Perera et al., 2013, 2014; Gebrekidan et al., 2015). Based on the sequence analysis of the major piroplasm surface protein (*MPSP*) gene, two (*chitose* and *ikeda*) of the 11 currently known genotypes (called *chitose* or *type* 1, *ikeda* or *type* 2, *buffeli* or *type* 3, *types* 4–8, and N1 to N3) of *T. orientalis* are considered to be pathogenic, leading to disease (oriental theileriosis) (reviewed by Sivakumar et al., 2014).

Although *T. orientalis* is known to be a cosmopolitan parasite of bovines (Uilenberg et al., 1985), the taxonomy of this complex is still controversial, and most of the studies describing various genotypes of *T. orientalis* have been carried out in the Asia-Pacific region, mainly utilizing the *MPSP* gene (reviewed by Sivakumar et al., 2014). To date, most of molecular studies focused on the characterization of the *T. orientalis* from the African continent have utilized the 18S rRNA gene using conventional PCR, followed by DNA sequencing (Allsopp et al., 1994; Collins et al., 2002; Darghouth, 2004; Salih et al., 2007; Altay et al., 2008; M'ghirbi et al., 2008; Chaisi et al., 2011, 2014). However, recently, Elsify et al. (2015) analysed *T. orientalis* for the first time using the *MPSP* gene from Africa, and reported *ikeda* and *chitose* genotypes from cattle and buffalo in Egypt.

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Very little is known about *T. orientalis* in cattle from Ethiopia. The first suspected cases of *T. orientalis* infection in cattle were reported in 1983; the parasite was detected using conventional (microscopic and serological) methods (Becerra et al., 1983), which are known to have limitations such as low specificity and sensitivity (cf. Perera et al., 2015a). Recently, Gebrekidan et al. (2014) detected *T. orientalis* in asymptomatic cattle in northern Ethiopia by conventional PCR, targeting the 18S rRNA gene. Since this study did not utilize the *MPSP* gene, *T. orientalis* could not be classified genetically as "pathogenic" or "non-pathogenic" genotypes (cf. Sivakumar et al., 2014). The present study detected, quantitated and characterized *T. orientalis* in local cattle in Ethiopia using PCR-based tools.

2. Materials and methods

2.1. Blood samples from cattle

A total of 232 blood samples randomly collected from clinically healthy/asymptomatic cattle were tested. Of these blood samples, 100 were available from a previous study carried out between Octo ber and November 2010 in three localities, including Addis Zemen (12° 7′ 11″N, 37° 46′ 48″E), Humera (14° 16′ 20″N, 36° 38′ 24″E) and Sheraro (14° 24′ 0″N, 37° 56′ 0″E) in northern Ethiopia, which were tested for various piroplasms (Gebrekidan et al., 2014). In addition, 132 more blood samples were collected in February 2013 from three more villages in Sheraro in northern Ethiopia.

Blood samples were taken from the jugular vein and collected into EDTA-coated tubes. The study was approved by the Ethiopian National Research Ethics Review Committee (NRERC; permit no. 3.10/3398/04). Consent was obtained from cattle owners for the collection of blood samples by an experienced, practicing veterinarian. Different cattle breeds were sampled in different locations, including Fogera and Zebu local breeds from Addis Zemen, and Arado and Barka (Begait) breeds from both Humera and Sheraro.

2.1.1. Isolation of DNA from blood, conventional PCR and DNA sequencing

Genomic DNA was extracted from 300 μL of each blood sample using the illustra blood genomicPrep Mini Spin Kit (GE HealthCare, Buckinghamshire, UK), following the manufacturer's instructions and stored at −20 °C until further analysis.

Primers MPSP-F and MPSP-R were used for the amplification of a 776 bp region of the *MPSP* gene as described previously (Ota et al., 2009). PCRs were conducted in 50 μL volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM $MgCl_2$, 6.25 μM of each deoxynucleotide triphosphate (dNTP), 100 pmol of each primer, and 1 U of Go*Taq* polymerase (Promega, USA). PCR cycling conditions were an initial denaturation at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and 72 °C for 5 min. Negative and positive controls were included in each PCR. PCR products were examined by agarose (1.5%) gel electrophoresis.

PCR products were then treated with shrimp alkaline phosphatase and exonuclease I (Germentas Inc., USA) (Werle et al., 1994), and subjected to bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, CA, USA) using (separately) the same primers employed in PCR. The quality of nucleotide sequences was appraised using the program Geneious Pro 5.6.5 (Biomatters Ltd., Auckland, New Zealand), and polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes. The *MPSP* sequences were identified by local alignment comparison (six reading frames) using amino acid sequences conceptually translated (using an online tool, http://www.ebi.ac.uk/Tools/st/emboss_transeq/) from reference sequences of *T. orientalis* available from the GenBank database.

2.1.2. Quantitative PCR

In order to estimate the infection intensity of *T. orientalis* genotypes present in Ethiopian cattle, we used a semiquantitative multiplex tandem PCR (MT-PCR) assay. This assay was conducted in the Easy-Plex platform (AusDiagnostics Pty Ltd, Australia) as described previously by Perera et al. (2015a) using primers designed specifically to the piroplasm surface protein (*p*23) gene (for genotype *buffeli*), *MPSP* gene (*chitose* and *type* 5) and the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA (*ikeda*) (cat. no. 38170R; AusDiagnostics, Australia). Following primary and secondary amplifications, the peak high resolution melting (HRM) temperature of each amplicon was compared with pre-determined reference temperatures representing individual genotypes: *buffeli* (83.6 ± 1.5 °C), *chitose* (82.1 ± 1.5 °C), *ikeda* (87.4 ± 1.5 °C) and *type* 5 (81.6 ± 1.5 °C) (Perera et al., 2015a). The relative intensity of infection of each of these four genotypes was expressed as a DNA copy number (Perera et al., 2015a). All amplicons had the peak melting temperatures within respective reference values. Amplicons from each genotype were subjected to single-strand conformation polymorphism (SSCP) analysis and targeted sequencing (Cufos et al., 2012; Perera et al., 2013).

2.1.3. Phylogenetic analyses

Nucleotide sequences were aligned using the MUSCLE V 3.8.31 program (Edgar, 2004) and adjusted manually employing the program Mesquite V 3.03 (Maddison and Maddison, 2008). Based on pairwise comparisons, sequence differences were calculated using the program MEGA 6.0. (Tamura et al., 2013).

A dataset representing the *MPSP* sequences detected in Ethiopia were compiled, together with key reference data from previous studies (Kawazu et al., 1992, 1999; Kubota et al., 1996; Kakuda et al., 1998; Kim et al., 1998; Gubbels et al., 2000; Zakimi et al., 2006; Altangerel et al., 2011a, b; Khukhuu et al., 2011; Sivakumar et al., 2013; Bawm et al., 2014; Elsify et al., 2015) available from the GenBank, with *T. annulata* (see Shiels et al., 1995) as the outgroup, and sequences were aligned (705 bp) and adjusted manually as described above. Phylogenetic analyses were performed using Bayesian Inference (BI) and Neighbor Joining (NJ) methods. The BI was conducted, using Monte Carlo Markov Chain (MCMC) analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood parameters for BI were based on the Akaike Information Criterion (AIC) test in jModeltest v2.1.5 (Darriba et al., 2012). The Tamura 3-parameter model of evolution, with gamma distribution and a proportion of invariable sites (T92 + Γ + I), was utilized for the analysis of the sequence data. The estimates of the base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100th tree being saved. A consensus tree (50% majority rule) was constructed based upon the remaining trees generated by BI. The NJ analyses were performed using the program MEGA 6.0, and the nodes were tested for robustness with 10,000 bootstrap replicates. The phylogenetic trees produced from the BI and NJ analyses were compared for concordance in their topologies.

2.1.4. Statistical analyses

Pearson's chi-square contingency Table analysis, Fisher's Exact Test and descriptive statistics were used to analyse the prevalence of genotypes detected by PCR and MT-PCR. DNA copy numbers (a measure of the intensity of infection) for the four *T. orientalis* genotypes were log-transformed (log10) and analysed by one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test. The SPSS Statistics 22 package (IBM) was used for

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