Ticks and Tick-borne Diseases 7 (2016) 291–297

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Population genetic structure of *Theileria parva* field isolates from indigenous cattle populations of Uganda

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ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form 31 October 2015
Accepted 12 November 2015
Available online 15 November 2015

Keywords:
Agro-ecological zones
Indigenous cattle populations
Infection and treatment method
Population genetic diversity
Theileria parva isolates
Uganda

ABSTRACT

Theileria parva causes East Coast Fever (ECF) a protozoan infection which manifests as a non-symptomatic syndrome among endemically stable indigenous cattle populations. Knowledge of the current genetic diversity and population structure of *T. parva* is critical for predicting pathogen evolutionary trends to inform development of effective control strategies. In this study the population genetic structure of 78 field isolates of *T. parva* from indigenous cattle (Ankole, $n = 41$ and East African shorthorn Zebu (EASZ), $n = 37$) sampled from the different agro ecological zones (AEZs) of Uganda was investigated. A total of eight mini- and micro-satellite markers encompassing the four chromosomes of *T. parva* were used to genotype the study field isolates. The genetic diversity of the surveyed *T. parva* populations was observed to range from 0.643 ± 0.55 to 0.663 ± 0.41 among the Central and Western AEZs respectively. The overall Wright's *F* index showed significant genetic variation between the surveyed *T. parva* populations based on the different AEZs and indigenous cattle breeds ($F_{ST} = 0.133$, $p < 0.01$) and ($F_{ST} = 0.101$, $p < 0.01$) respectively. Significant pairwise population genetic differentiations ($p < 0.05$) were observed with F_{ST} values ranging from 0.048 to 0.173 between the eastern and northern, eastern and western populations respectively. The principal component analysis (PCA) showed a high level of genetic and geographic sub-structuring among populations. Linkage disequilibrium was observed when populations from all the study AEZs were treated as a single population and when analysed separately. On the overall, the significant genetic diversity and geographic sub-structuring exhibited among the study *T. parva* isolates has critical implications for ECF control.

1. Introduction

Theileria parva is a protozoan parasite which causes East Coast Fever (ECF), a deadly lympho-proliferative disease of mainly exotic but also indigenous cattle (Waladde et al., 1993) in subSaharan Africa (SSA). East Coast Fever (ECF) conflicts with several national plans to improve cattle productivity in the region (Morrison et al., 1987). The disease discourages hybridisation of indigenous cattle with exotic genotypes which are highly susceptible to the infection and yet the latter are desired for higher productivity (Conelly, 1998; Gachohi et al., 2012). Although this disease causes about one million deaths every year, another 28 million are at constant risk of getting infected (Morrison et al., 1987).

The use of acaricides to check tick infestations on cattle is the most common approach for controlling tick-borne diseases, especially ECF. This method has been recently disadvantaged by acaricide resistant tick populations, food-safety concerns and environmental pollution (George et al., 2004). One of the approaches envisaged to curtail the development of acaricide resistant tick populations is the use of infection and treatment method (ITM) vaccination which currently uses the live Muguga cocktail vaccine being popularised for integrated ECF control in eastern Africa (Di Giulio et al., 2009; GALVmed, 2011). The ITM was developed (Radley et al., 1975) to mimic the acquisition of natural immunity developed among indigenous cattle after primary exposure to ECF. It was shown that using a mixture of three East African isolates (Kiambu 5, Muguga and Serengeti transformed), a high degree of protection could be achieved in an area with field strains arriving from many parts of Tanzania (Uilenberg et al., 1977, 1978). Its widespread deployment is nevertheless constrained by concerns of possible introduction of novel parasite genotypes to unvaccinated

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http://dx.doi.org/10.1016/j.ttbdis.2015.11.004
1877-959X/

cattle (De Deken et al., 2007; Oura et al., 2007), via vaccine-derived infected ticks. In addition, it has been perceived that *T. parva* can undergo population genetic transformation in the short-term after ITM vaccination (Bishop et al., 2001; Oura et al., 2004, 2007; Mwega et al., 2014). The occurrence of ECF outbreak among vaccinated cattle due to challenge infection with different parasite strains from the vaccine stabilates might therefore demands additional combination of local strains into the vaccine (Sitt et al., 2015).

Since indigenous cattle are reared in all the AEZs of Uganda and are often non-clinical carriers of *T. parva*, they can be a constant source of infection to naïve exotic cattle (Kabi et al., 2014). Additionally, the unlimited movement of *T. parva* infected cattle might introduce novel parasite genotypes to new locations (Geysen et al., 1999; De Deken et al., 2007; Hayashida et al., 2013; Mwega et al., 2014). This will continuously complicate the population genetic structure of *T. parva* and hinder the use of ITM in the control of ECF. Location specific studies of *T. parva* field isolates have demonstrated genetically diverse parasite populations in Kenya (Nene et al., 1992), Zimbabwe (Bishop et al., 1994), Uganda (Oura et al., 2011) and Zambia (Muleya et al., 2012).

Mini and micro-satellite markers have been validated for revealing the genetic diversity, population dynamics and geographic origins of *T. parva* field isolates (Oura et al., 2003, 2005; Katzer et al., 2006; Odongo et al., 2006). Although *T. parva* genetic diversity location specific studies have been conducted in the recent past (Oura et al., 2011), this survey provides additional information on nationwide population genetic structure with comparisons between contrasting agro-ecological zones (AEZs) and indigenous cattle breeds to further allow strategic planning of ECF control integrated with the impending ITM approach in Uganda.

2. Materials and methods

2.1. Sampling, genomic DNA extraction and nested PCR detection of T. parva

Blood samples were collected from indigenous cattle breed groups (Ankole and EASZ) with no apparent clinical signs of ECF in a landscape approach throughout the entire country as previously described in Kabi et al. (2014).

A total of 78 DNA samples, which previously tested positive in a nested polymerase chain reaction (nPCR) assay for *T. parva* using the p104 (Odongo et al., 2010) marker were used for this investigation. The samples were categorised according to their origin including: Central – Lake Victoria crescent ($n = 11$), Eastern – North eastern savannah grasslands and drylands ($n = 18$), Northern – Northwestern savannah grasslands ($n = 19$) and Western – Pastoral Rangelands, South Western Highland Ranges and Western Savannah grasslands ($n = 30$) AEZs of Uganda as shown in Fig. 1.

2.2. PCR amplification and analysis of mini- and microsatellite loci

In this study, a total of eight mini- and two microsatellite loci previously described by Oura et al. (2003), and known to be polymorphic were used. At most, two markers per chromosome were chosen on each of the four chromosomes of *T. parva* as shown in Table 1. A total of 78 samples were genotyped with a total of eight loci. In the nested PCR, the forward primers of each primer pair were fluorescently labelled at the 5′-end with one of the following four standard dyes; 6-FAM (Blue), NED (Yellow), PET (Red) and VIC (Green) (Bioneer, Korea), for detection on an ABI genetic analyser. Genomic DNA (10 ng) were amplified using 5 U *Taq* polymerase (Promega), 1× PCR buffer (10 mM Tris–HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$), 0.2 mM dNTPs, and 0.2 μM of each reverse and forward primers, and made up to a final reaction volume of 10 μl with sterile water. The PCR was carried out in a Gene Amp® PCR System 9700 (ABI-USA). The cycling conditions were optimised as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min and final extension was at 72 °C for 20 min before cooling to 4 °C. The reaction was run for 35 cycles.

To prepare for capillary electrophoresis a reaction mix containing (0.5–2 μl) of PCR product was added into each well of a 96-well plate containing 8 μl of Hi-Di Formamide and GeneScan 500 LIZ size standard (ABI-USA). Rapid denaturation was carried out in a thermocycler at 95 °C for 5 min followed by rapid chilling of the micro-titre plate on ice. The ABI 3730 genetic analyser (Applied Biosystems-USA) was used to analyse the amplicons. The alleles were scored using the Gene-Mapper programme (Applied Biosystems-USA). Amplicons with a maximum peak height were scored, and a predominant peak was defined as that with the largest area under the curve. All data generated from the Gene-Mapper were resized by the allelobin software based on consensus sequence repeats of the markers. A predominant allele at each locus was used to generate allele frequency data and multilocus genotypes (MLGs) in an excel file.

2.3. Data analyses

Summary statistics of allele frequencies per locus within each *T. parva* population was calculated using the genetic analysis package Power Marker V. 3.25 (Liu and Muse, 2005). Major allele frequency, gene diversity (GD) and polymorphic information content (PIC) were used to determine diversity at each mini- and microsatellite locus in each of the *T. parva* populations from the eastern, central, northern and western AEZs zones. The maximum number of alleles identified for each marker, patterns of genotypic distribution in each study populations, genotype diversity and analysis of molecular variance (AMOVA) among and within populations was estimated by GenAlEx V. 6.5 (Peakall and Smouse, 2006) add-in microsoft excel software. The statistical significance of AMOVA was assessed using 999 random permutations. In addition the principal component analysis (PCA) was used for comparison of the different *T. parva* populations based on the different AEZs.

The null hypothesis of linkage equilibrium was tested using LIAN an online software (http://adenine.biz.fh-weihenstephan.de/lian/) which computes the standardised index of association (I_A^S) and quantifies linkage equilibrium (LE) or disequilibrium (LD) as previously defined by Haubold and Hudson (2000). The I_A^S measures the association between alleles at pairs of loci, i.e., I_A^S values close to 0 or negative is indicative of panmixia, while those significantly greater than 0 is indicative of non-panmictic populations. Linkage equilibrium is characterised by the statistical independence of alleles across all loci and is investigated by initially determining the number of loci at which each pair of multi-locus genotypes (MLGs) differs. From the distribution of mismatch values, a variance (V_D) is calculated which is compared to the variance expected for LE, termed V_e. The null hypothesis that $V_D = V_e$ is tested by both a Monte Carlo simulation and a parametric method in order to estimate a 95% confidence limit (95% CI), which are denoted L_{mc} and L_{para}, respectively. When V_D is found to be greater than L, the null hypothesis is rejected and LD is accepted.

2.4. Ethical clearance

This study was ethically cleared by Makerere University Institute of Environment and Natural Resources (MUIENR) and approved by the higher degree committee of Makerere University. Permission to undertake the study was obtained from the Uganda National Council for Science and Technology (UNCST) reference