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Research paper

Theileria lestoquardi displays reduced genetic diversity relative to sympatric *Theileria annulata* in Oman

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ABSTRACT

The Apicomplexan parasites, *Theileria lestoquardi* and *Theileria annulata*, the causative agents of theileriosis in small and large ruminants, are widespread in Oman, in areas where cattle, sheep and goats co-graze. Genetic analysis can provide insight into the dynamics of the parasite and the evolutionary relationship between species. Here we identified ten genetic markers (micro- and mini-satellites) spread across the *T. lestoquardi* genome, and confirmed their species specificity. We then genotyped *T. lestoquardi* in different regions in Oman. The genetic structures of *T. lestoquardi* populations were then compared with previously published data, for comparable panels of markers, for sympatric *T. annulata* isolates. In addition, we examined two antigen genes in *T. annulata* (*Tams1* and *Ta9*) and their orthologues in *T. lestoquardi* (*Tlms1* and *Tl9*).

The genetic diversity and multiplicity of infection (MOI) were lower in *T. lestoquardi* (H_e = 0.64–0.77) than *T. annulata* (H_e = 0.83–0.85) in all populations. Very limited genetic differentiation was found among *T. lestoquardi* and *T. annulata* populations. In contrast, limited but significant linkage disequilibrium was observed within regional populations of each species. We identified eight *T. annulata* isolates in small ruminants; the diversity and MOI were lower among ovine/caprine compared to bovine. Sequence diversity of the antigen genes, *Tams1* and *Ta9* in *T. annulata* ($\pi = 0.0733$ and $\pi = 0.155$ respectively), was 10-fold and 3-fold higher than the orthologous *Tlms1* and *Tl9* in *T. lestoquardi* ($\pi = 0.006$ and $\pi = 0.055$, respectively).

Despite a comparably high prevalence, *T. lestoquardi* has lower genetic diversity compared to sympatric *T. annulata* populations. There was no evidence of differentiation among populations of either species. In comparison to *T. lestoquardi*, *T. annulata* has a larger effective population size. While genetic exchange and recombination occur in both parasite species, the extent of diversity, overall, is less for *T. lestoquardi*. It is, therefore, likely that *T. lestoquardi* evolved from an ancestor of present day *T. annulata* and that this occurred either once or on a limited number of occasions.

1. Introduction

Theileria lestoquardi is a highly pathogenic ovine and caprine parasite and is considered to be the only *Theileria* species of economic significance in small ruminants (Leemans et al., 2001; Li et al., 2014). The parasite is transmitted by *Hyalomma anatolicum anatolicum*, which is common in South-eastern Europe, Northern Africa, Southern Russia and the Middle East. However, distribution of *T. lestoquardi* is limited compared to the range of its vector. Although *T. lestoquardi* has been shown to be antigenically closely related to *T. annulata* (Leemans et al., 1997), it has been reported as being incapable of infecting cattle (Leemans et al., 1999). Conversely, it is known that *T. annulata* can infect sheep; experiments in sheep indicate that *T. lestoquardi* infection protects against subsequent *T. annulata* infection (Leemans et al., 1999) and although prior infection with *T. annulata* does not prevent infection from *T. lestoquardi* sporozoites, it does protect against the major clinical effects. However, these experiments were carried out on limited numbers of animals with a very limited number of parasite genotypes, and the actual transmission dynamics in the field are unknown.

T. lestoquardi was first reported in sheep in Sudan and Egypt (Littlewood, 1916), and later detected in sheep and goats in other

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countries of the Middle East such as Algeria (Lestoquard, 1927), Turkey (Baumann, 1939), Iraq (Khayyat and Gilder, 1947), Iran (Hooshmand-Rad and Maghami, 1976; Hawa et al., 1981), as well as India (Raghvachari and Reddy, 1959) and Serbia (Dschunkovsky and Urodschevich, 1924). A previous study in Oman demonstrated a high level of theileriosis-attributed mortality in a local sheep breed (Tageldin et al., 2005). This confirmed previous individual case reports and outbreak records of a pathogenic species of *Theileria* in sheep and goats in Oman (Annual Reports VRC2004–2006) (MOAF, 2008). These reports indicated that in Oman, sheep, in general, were significantly more at risk of clinical theileriosis than cattle and goats, and this has been attributed to a higher tick infestation of sheep. However, the relative distribution of the major pathogenic species of *Theileria* (*T. lestoquardi* and *T. annulata*) is not yet known in the Sultanate of Oman. Thus, there is currently no information on the prevalence of *T. lestoquardi* in different regions in Oman and nothing is known regarding the *T. lestoquardi* population structure. In contrast, a recent survey demonstrated that *T. annulata* is widely distributed across the country and is comprised of a highly genetically diverse, inter-breeding population (Al-Hamidhi et al., 2015).

Genetic analysis of parasite populations can provide important information about the epidemiology of disease and may facilitate the development of rational control approaches. Polymorphic genetic markers have been developed for some species of *Theileria*, e.g. *T. annulata* and *T. parva* (Oura et al., 2003; Weir et al., 2007), however, such tools are not yet available for the small ruminant *Theileria* species parasites, *T. lestoquardi* and *T. ovis*. Micro- and mini-satellites are considered as highly appropriate molecular markers for population genetic applications. Their high mutation rate and Mendelian mode of inheritance make them particularly useful for the study of both fine and broad-scale population genetic structure (Abdelkrim et al., 2009). Common applications include assessing genetic diversity, degree of population inbreeding, bottleneck effects, gene flow and migration rates, the assignment of population of origin and parental lineages (Goldstein and Schlotterer, 1999).

The present study included the development of micro- and mini-satellite genotyping for *T. lestoquardi* and their application to investigate the genetic diversity of parasite populations from four regions in Oman. The extent of diversity and population structure of *T. lestoquardi* was then compared to available published data on sympatric *T. annulata* populations for three of the four regions. We aimed to gain an understanding of whether local gene flow and genetic diversity differs between these two species in an area of similar prevalence and distribution of tick species. We also investigated the hypothesis that *T. lestoquardi* is a relatively recently evolved species that has diverged from the more ancient cattle parasite species, *T. annulata*, following a host species jump to small ruminants.

2. Materials and method

2.1. Parasite material and DNA preparation

Blood samples ($n = 1454$) were collected from clinically healthy sheep and goats in four governorates of Oman: Batinah ($n = 584$), Dhira ($n = 357$), Sharqia ($n = 369$) and Dakhiliya ($n = 144$) (Fig. 1). The climate across these regions is hot and dry throughout the year, with 3–4 months (Oct to Feb) of relatively moderate temperatures (below 30 °C).

For comparison of diversity and population structure, genotyping data representing 97 *T. annulata* isolates from Batinah ($n = 21$), Dhira ($n = 57$) and Sharqia ($n = 19$) derived from cattle co-grazed on the same farms as the sheep/goats that provided *T. lestoquardi* isolates was utilised. These were previously genotyped with a set of *T. annulata* specific micro- and mini-satellites (Al-Hamidhi et al., 2015).

2.2. Identification of specific T. lestoquardi micro- and mini-satellite sequences

A draft sequence of the *T. lestoquardi* genome has been generated (Weir et al., unpublished). To identify micro- and mini-satellite loci specific for *T. lestoquardi*, sequence contigs were screened using the tandem repeat finder program (Benson, 1999). A filtration pipeline was used to identify a subset of high-value loci, which could be tested using a panel of available stocks and isolates. Filtration included discarding repeat regions >500 bp in length and those that possessed insufficient flanking sequence for primer design. The remaining sequences were ranked, based on the fidelity of the repeat within each region (>70% fidelity) and the number of repeats. A subset of 28 loci with conserved repeat motifs was then derived.

2.3. PCR amplification of specific micro- and mini-satellite loci

Primers were designed to unique sequence flanking each repeat and used to amplify DNA purified from a panel of stocks (*T. lestoquardi*, *T. annulata* and *T. ovis*) and field isolates to test marker specificity and polymorphism. In addition, to test for marker sensitivity, serial dilutions of *T. lestoquardi* DNA were generated and PCR performed with each primer set and sample.

PCR was carried out in a total reaction volume of 20 μl using conditions described previously (Al-Hamidhi et al., 2015). Thermocycler parameters were as follows: denaturation at 94 °C for 5 min, 32 cycles at 94 °C for 30 s, 42–55 °C for 30 s, and 65 °C for 30 s, followed by a final extension step of 5 min at 65 °C. Amplified products were observed on a 2% ethidium bromide pre-stained agarose gel and their size determined with reference to either a 1 kb or 100 bp DNA ladder.

To identify length polymorphism down to the level of 1 base pair (bp), PCR products were denatured and then capillary electrophoresed in an ABI3130 xl Genetic Analyser (Applied Biosystems, UK). DNA fragment sizes were determined relative to ROX-labeled GS500 size-standards (Applied Biosystems) using GeneMapper software (Applied Biosystems). For all loci and DNA samples, fragment size (i.e. peak position) was determined to two decimal places. Analysis of the distribution of fragment sizes facilitated the creation of 'fixed bins' of variable sizes to score alleles. Since these loci represent genomic regions encoding hypothetical proteins, variation among allele sizes was assumed to be in steps of three base pairs or multiples thereof.

The single or predominant allele for each of the ten selected loci was utilised to compute allele frequencies. Each of the markers selected for further analysis was shown to represent a different single-copy locus based on genome data and PCR fragments amplified from *T. lestoquardi* (Lahr) DNA. Since *Theileria* parasites are haploid, the presence of one or more additional alleles at a particular locus was interpreted as a co-infection with one or more genetically distinct genotypes. An additional allele was scored if the peak was at least one-third the height of the predominant allele (highest peak) on the electropherogram traces, a method that has been widely used in previous studies (Anderson et al., 1999). In this way, the predominant allele at each locus was identified for each sample and the data combined to generate a multi-locus genotype (MLG), representing an estimate of the most abundant genotype in each sample, as described previously (Weir et al., 2007).

The MLG dataset was then used to measure population genetic indices such as heterozygosity, linkage disequilibrium and population differentiation. Since *Theileria* is haploid and heterozygosity cannot be observed directly, the estimated heterozygosity was calculated using the predominant allele dataset for each marker and averaged across all ten loci.