Evidence for Extensive Genetic Diversity and Substructuring of the Babesia bovis Metapopulation

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Summary

Babesia bovis is a tick-transmitted haemoprotozoan and a causative agent of bovine babesiosis, a cattle disease that causes significant economic loss in tropical and subtropical regions. A panel of nineteen micro- and minisatellite markers was used to estimate population genetic parameters of eighteen parasite isolates originating from different continents, countries and geographic regions including North America (Mexico, USA), South America (Argentina, Brazil), the Middle East (Israel) and Australia. For eleven of the eighteen isolates, a unique haplotype was inferred suggesting selection of a single genotype by either in vitro cultivation or amplification in splenectomized calves. Furthermore, a high genetic diversity (H = 0.780) over all marker loci was estimated. Linkage disequilibrium was observed in the total study group but also in sample subgroups from the Americas, Brazil, and Israel and Australia. In contrast, corresponding to their more confined geographic origin, samples from Israel and Argentina were each found to be in equilibrium suggestive of random mating and frequent genetic exchange. The genetic differentiation (FST) of the total study group over all nineteen loci was estimated by analysis of variance (Θ) and Nei’s estimation of heterozygosity (GST) as 0.296 and 0.312, respectively. Thus, about 30% of the genetic diversity of the parasite population is associated with genetic differences between parasite isolates sampled from the different geographic regions. The pairwise similarity of multilocus genotypes (MLGs) was assessed and a neighbour-joining dendrogram generated. MLGs were found to cluster according to the country/continent of origin of isolates, but did not distinguish the attenuated from the pathogenic parasite state. The distant geographic origin of the isolates studied allows an initial glimpse into the large extent of genetic diversity and differentiation of the B. bovis population on a global scale.

Introduction

Bovine babesiosis is a major impediment to cattle farming in tropical and subtropical regions around the world. Babesia bovis is one of the most virulent causative agents of the disease associated with high mortality (Bock et al., 2004; Schnittger et al., 2012). About 500 million cattle heads are raised in endemic areas and at risk of infection (Bock et al., 2004; Gohil et al., 2012). Animals that survive the acute phase of infection develop protective immunity against field
tick challenge; however, they remain lifelong parasite carriers. Parasitaemia in these chronically infected animals is extremely low and fluctuating, and even when highly sensitive molecular diagnostic tools such as nested PCR are used, the parasite may escape detection (Calder et al., 1996; Gubbels et al., 1999). This may potentially complicate PCR-based typing of field isolates using micro- and minisatellite markers, and the commonly observed high multiplicity of infection might obscure correct assignment of alleles to their respective haplotypes. To circumvent this difficulty, the present study mainly included *B. bovis* isolates that have been subjected to *in vitro* cultivation and/or amplification in spleenectomized calves. These isolates provide the additional advantage of their replenishable genomic DNA and are commonly referred to as reference isolates. Many of these isolates have been attenuated by multiple passages in spleenectomized calves to be used as vaccines in countries where vaccination is a common control measure such as Argentina, Australia, Brazil and Israel (Shkap et al., 2007).

Multilocus genotyping using micro- and minisatellites may provide important insights into the genetic diversity and structure of *B. bovis* populations (Beck et al., 2009; Schnittger et al., 2012). Recently a set of micro- and minisatellite markers has been developed and applied to analyse five *B. bovis* reference strains originating from different geographic regions of the Americas (Perez-Llaneza et al., 2010). The established MLG differences of these strains could be linked to their geographic origin. To further substantiate the notion of geographic substructuring, a more extensive number of marker loci (19 versus 14) were used to study a considerably larger number of *B. bovis* reference strains (18 versus 5) originating from various distant countries and continents around the world. In addition to the estimation of genetic differentiation, the genetic and haplotype diversity as well as the pairwise linkage disequilibrium between marker loci were assessed.

### Material and Methods

Designation, geographic origin and characteristics of parasite stocks included in this study are shown in Table 1. Parasite stocks designated B7A (Brazil), I1A and I3A (Israel), and R1A and M1A (Argentina) are used as live vaccines in their country of origin. All parasite stocks except for the virulent field isolate I2P Avigdor, Israel, have either been cultured *in vitro* or amplified in spleenectomized calves prior to isolation of genomic DNA using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Extracted DNA was amplified by PCR as described in Perez-Llaneza et al. (2010). Primers used to amplify micro- and minisatellite markers are described in Perez-Llaneza et al. (2010), Simuunza et al. (2011) and Flores et al. (2011). Amplification products were separated on 5% polyacrylamide/7M urea denaturing gels and detected by silver staining (Bio-Rad, Hercules, CA). A DNA ladder was used to estimate the size of each band (Invitrogen, Carlsbad, CA, USA). Letters were assigned in alphabetical order with increasing size of allelic bands, and the MLG of each individual sample determined. Linkage equilibrium is characterized by statistical independence of alleles at all pairwise combinations of loci under investigation. The null hypothesis of linkage equilibrium (LE) was tested, and the standard index of association (**I**<sub>A</sub>) calculated using LIAN (Haubold and Hudson, 2000). A standard **I**<sub>A</sub> of zero or close to zero indicates LE, while higher values quantify linkage disequilibrium (LD). In addition, the significance level of rejection of LE is assessed by Monte Carlo simulation. LIAN was also used to determine the mean genetic diversity (**H**) over all studied loci. The null hypothesis (**H**<sub>0</sub>; samples were drawn from the same population) was tested by an exact G-test using GenePop (Raymond and Rousset, 1995). The population genetic parameters **Θ** (Theta), and **G**<sub>ST</sub> and standard deviation of **Θ** were determined by F-statistics using FSTAT version 2.9.3.2 (Weir and Cockerman, 1984; Nei, 1987; Goudet, 1995). A similarity matrix was established by pairwise comparison of MLGs, and a dendrogram generated using neighbour joining with the Web-based application Clustering Calculator (http://www2.biology.ualberta.ca/jbrzusto/cluster.php) (Saitou and Nei, 1987).

### Results and Discussion

Determination of 18 different MLGs in the study group (**n** = 18 isolates) is indicative of a high level of genetic diversity. This observation was not surprising, as an extensive diversity of MLGs has been reported even in geographically more confined population genetic analyses of *B. bovis*, and its close relatives, *Theileria annulata* and *T. parva* (Oura et al., 2003, 2005; Odongo et al., 2006; Weir et al., 2007; Perez-Llaneza et al., 2010). Also, when large numbers of parasite isolates have been investigated, repetitive MLGs were relatively rarely observed (Weir et al., 2011; Schnittger et al., 2012). After all, in a total of 120 analysed *B. bovis* isolates, Simuunza et al. (2011) observed exclusively unique MLGs.

In the study group, the individual genetic diversity (**H**) of marker loci varied between 0.484 and 0.922, while the mean genetic diversity (**H**) across all 19 loci was estimated as 0.780. The genetic diversity of *B. bovis* has recently been estimated 0.834 and 0.837 in Zambia and Turkey, respectively (Simuunza et al., 2011). The lower genetic diversity in our study group may be attributed to the inclusion of additional marker loci, some of which exhibit a lower allelic polymorphism. Simuunza et al. (2011) employed eight highly variable micro- and minisatellite markers. When our
estimation is based on the eight most polymorphic of the nineteen markers applied in the present study, the mean genetic diversity resulted in a slightly higher estimate of 0.864, compared with that determined for Zambia or Turkey.

On average, six different alleles, varying in a range from three to nine, were identified per marker loci in the study group. In field isolates, multiple alleles of a single marker locus are commonly detected and are indicative for the existence of multiple parasite genotypes in a single animal (Simuunza et al., 2011; Weir et al., 2011). In contrast, in the present study group, a single allele and genotype was detected in eleven of eighteen isolates, suggesting the selection of the variant erythrocyte surface 1 (ves) multigene family (Lau et al., 2011). It has been suggested that subpopulation selection may be a mechanism of attenuation (Baravalle et al., 2011). It has been proposed that selection among parasite variants in amplified virulent as well as in attenuated isolates.

Linkage equilibrium between alleles at pairs of loci was tested to determine whether the study group either constitutes a panmictic (randomly mating) or a non-panmictic population. A standard index of association $I_A$ (0 ≤ $I_A$ ≤ 1) was estimated as a quantitative measure of an increasing strength of linkage. An $I_A$ is equal or close to zero represents LE and therefore panmixis, while larger values of $I_A$ indicate LD ($I_A = 1$ is indicative for complete linkage). As shown in Table 2, the study group displays a highly significant LD with a standardized $I_A$ of 0.1203. The observation of LD may be due to geographic substructuring of a population. To test this possibility, samples were pooled according to their origin and retested. A highly significant LD was detected in sample groups originating from the Americas ($n = 12$), Brazil ($n = 6$), and Israel and Australia ($n = 6$), while samples originating from Argentina ($n = 4$) and Israel ($n = 4$) were found to be in LE (Table 2). This suggests that samples originating from the latter two countries possibly pertain each to a confined parasite population that exhibits frequent recombination and is unstructured.

Indeed, the significant closer distance between the geographic origin of samples from Argentina and Israel, respectively, than between samples originating from other countries/regions corroborates this finding. It seems to be the most reasonable explanation to assume geographic substructuring as a reason for the observed LD within samples from the Americas and Brazil, respectively, as within those vast continents/countries, the samples originated from distant geographic regions (Table 1). Notwithstanding, the observation of LE within the Israel sample and of LD within the joint group of Israel and Australian samples (standard $I_A = 0.2134$) is indicative of a geographic substructuring between these two countries. Although

<table>
<thead>
<tr>
<th>Name (isolate)</th>
<th>Country and region of origin</th>
<th>Characteristics</th>
<th>Reference (or provider)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2Bo-P</td>
<td>Texas, USA</td>
<td>Pathogenicb</td>
<td>Hines et al. (1992)</td>
</tr>
<tr>
<td>Mo7-P</td>
<td>Mexico</td>
<td>Pathogenicb</td>
<td>Rodriguez et al. (1983), Shkap et al. (1994)</td>
</tr>
<tr>
<td>R1A</td>
<td>Santa Fe, Argentina</td>
<td>Attenuatedb</td>
<td>Anziani et al. (1993)</td>
</tr>
<tr>
<td>S2P</td>
<td>Salta, Argentina</td>
<td>Pathogenicb</td>
<td>Echaide et al. (1993)</td>
</tr>
<tr>
<td>M1A</td>
<td>Salta, Argentina</td>
<td>Attenuated</td>
<td>Daniel Benitez</td>
</tr>
<tr>
<td>M2P</td>
<td>Corrientes, Argentina</td>
<td>Pathogenic</td>
<td>Daniel Benitez</td>
</tr>
<tr>
<td>B2P</td>
<td>Bahia, Brazil</td>
<td>Pathogenic</td>
<td>Flabio R. Araujo, Ramos et al. (2012)</td>
</tr>
<tr>
<td>B3P (6409)</td>
<td>Mato Grosso do Sul, Brazil</td>
<td>Pathogenic</td>
<td>Flabio R. Araujo, Ramos et al. (2012)</td>
</tr>
<tr>
<td>B5P (7271)</td>
<td>Mato Grosso do Sul, Brazil</td>
<td>Pathogenic</td>
<td>Flabio R. Araujo, Ramos et al. (2012)</td>
</tr>
<tr>
<td>B6P</td>
<td>Rio Grande do Sul, Brazil</td>
<td>Pathogenic</td>
<td>Flabio R. Araujo, Ramos et al. (2012)</td>
</tr>
<tr>
<td>B7A</td>
<td>EMBRAPA, Campo Grande, Mato Grosso do Sul, Brazil</td>
<td>Attenuated</td>
<td>Flabio R. Araujo, Ramos et al. (2012)</td>
</tr>
<tr>
<td>A1P (F71 plus 1)</td>
<td>North Queensland, Australia</td>
<td>Unattenuatedc</td>
<td>Bock et al. (1995)</td>
</tr>
<tr>
<td>A14P (H66)</td>
<td>Central, Queensland, Australia</td>
<td>Pathogenic</td>
<td>Bock et al. (1992, 1995)</td>
</tr>
<tr>
<td>I1A</td>
<td>Mevo Horon, Israel</td>
<td>Attenuated</td>
<td>Mazuz et al. (2012)</td>
</tr>
<tr>
<td>I2 P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Avigdor, Israel</td>
<td>Pathogenic</td>
<td>Varda Shkap</td>
</tr>
<tr>
<td>I3A (725)</td>
<td>Gonen, Israel</td>
<td>Attenuated</td>
<td>Mazuz et al. (2012)</td>
</tr>
<tr>
<td>I4P (T63)</td>
<td>Katcha, Israel</td>
<td>Pathogenic</td>
<td>Mazuz et al. (2012)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isolate of pathogenic field isolate of an acute babesiosis case from Avigdor, Israel, that has neither been cultured nor amplified in splenectomized calves.

<sup>b</sup> Isolate has been cultured in vitro.

<sup>c</sup> Attenuated isolate has been cultured in vitro.
Table 2. Analysis of linkage equilibrium between pairwise loci

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Number</th>
<th>$I_A$ (standardized)</th>
<th>Significance</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>18</td>
<td>0.1203</td>
<td>&lt;0.001</td>
<td>LD</td>
</tr>
<tr>
<td>The Americas</td>
<td>12</td>
<td>0.1264</td>
<td>&lt;0.001</td>
<td>LD</td>
</tr>
<tr>
<td>Brazil</td>
<td>6</td>
<td>0.1164</td>
<td>&lt;0.001</td>
<td>LD</td>
</tr>
<tr>
<td>Argentina</td>
<td>4</td>
<td>0.0037</td>
<td>ns</td>
<td>LE</td>
</tr>
<tr>
<td>Israel and Australia</td>
<td>6</td>
<td>0.2134</td>
<td>&lt;0.001</td>
<td>LD</td>
</tr>
<tr>
<td>Israel</td>
<td>4</td>
<td>0.0052</td>
<td>ns</td>
<td>LE</td>
</tr>
</tbody>
</table>

$I_A$ (standardized), index of standardized association between pairs of loci; LE, linkage equilibrium; LD, linkage disequilibrium; ns, non significant.

Fig. 1. Dendrogram of pairwise similarity of *B. bovis* isolates. A, attenuated strains; P, pathogenic strains.

Table 2 suggests that the Argentinean parasite subgroup exhibits a lower level of differentiation than those from other regions. However, a larger sample size will be necessary to verify this notion. Importantly, while clustering of MLGs is predictive for the country/continent origin of studied isolates, it does not predict the attenuated versus the pathogenic parasite state. Thus, either our markers are not linked to attenuation factors, or attenuation may be a characteristic which is not associated with a single genetic factor but with more complex multifactorial parameters of the parasite genome (Lau et al., 2011).

In summary, the study group of *B. bovis* reference isolates showed a high diversity of marker loci and MLGs, indicative of genetic recombination and random assortment of alleles. LE was observed only for samples originating from more confined regions from Argentina and Israel, while the total study group displayed significant LD suggesting geographic substructuring. This was further corroborated by a very high genetic differentiation of the study group, and the observation that *B. bovis* isolates segregated into clusters according to their country/continent of origin. These findings may have important implications for vaccine efficiency in different geographic regions and the dissemination of genetic resistance factors in the parasite population.

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Conflicts of interest

The authors declare no conflicts of interest in relation to this work.

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