Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

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 (F_{ST}) of the total study group over all nineteen loci was estimated by analysis of variance (Θ) and Nei's estimation of heterozygosity ($G_{ST'}$) 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 splenectomized 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 splenectomized 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 splenectomized 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_A) calculated using LIAN (Haubold and Hudson, 2000). A standard I_A 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_0: samples were drawn from the same population) was tested by an exact G-test using Genepop (Raymond and Rousset, 1995). The population genetic parameters Theta (Θ), and G_{ST}' and standard deviation of Θ were determined by F-statistics using FSTAT version 2.9.3.2 (Weir and Cockerham, 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

Table 1. *Babesia bovis* isolates

Name (isolate)	Country and region of origin	Characteristics	Reference (or provider)
T2Bo-P	Texas, USA	Pathogenic[b]	Hines et al. (1992)
Mo7-P	Mexico	Pathogenic[b]	Rodriguez et al. (1983), Shkap et al. (1994)
R1A	Santa Fe, Argentina	Attenuated[b]	Anziani et al. (1993)
S2P	Salta, Argentina	Pathogenic[b]	Echaide et al. (1993)
M1A	Salta, Argentina	Attenuated	Daniel Benitez
M2P	Corrientes, Argentina	Pathogenic	Daniel Benitez
B2P (5796)	Bahia, Brazil	Pathogenic	Flabio R. Araujo, Ramos et al. (2012)
B3P (6409)	Mato Grosso do Sul, Brazil	Pathogenic	Flabio R. Araujo, Ramos et al. (2012)
B4P (6568)	Rio Grande do Sul, Brazil	Pathogenic	Flabio R. Araujo, Ramos et al. (2012)
B5P (7271)	Mato Grosso do Sul, Brazil	Pathogenic	Flabio R. Araujo, Ramos et al. (2012)
B6P	Rio Grande do Sul, Brazil	Pathogenic	Flabio R. Araujo, Ramos et al. (2012)
B7A	EMBRAPA, Campo Grande, Mato Grosso do Sul, Brazil	Attenuated	Flabio R. Araujo, Ramos et al. (2012)
A1P (F71 plus 1)	North Queensland, Australia	Unattenuated[c]	Bock et al. (1995)
A14P (H66)	Central, Queensland, Australia	Pathogenic	Bock et al. (1992, 1995)
I1A	Mevo Horon, Israel	Attenuated	Mazuz et al. (2012)
I2 P[a]	Avigdor, Israel	Pathogenic	Varda Shkap
I3A (725)	Gonen, Israel	Attenuated	Mazuz et al. (2012)
I4P (T63)	Katcha, Israel	Pathogenic	Mazuz et al. (2012)

[a]I2P, pathogenic field isolate of an acute babesiosis case from Avigdor, Israel, that has neither been cultured nor amplified in splenectomized calves.
[b]These isolates have been cultured *in vitro*.
[c]The pathogenicity of the original field isolate has been described as only mildly pathogenic.

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 *B. bovis* genotypes by *in vitro* culture and also by parasite amplification in splenectomized calves. This observation is in line with recent reports were parasite attenuation was found to be associated with a strong overall reduction in genome diversity, as well as a reduced diversity 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., 2012). However, as determined by sequencing of the polymorphic Bv80 gene, Mazuz et al. (2012) found various 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 ($0 \leq I_A \leq 1$) 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 2. Analysis of linkage equilibrium between pairwise loci

Sample group	Number (*n*)	I_A (standardized)	Significance	Linkage
Total	18	0.1203	<0.001	LD
The Americas	12	0.1264	<0.001	LD
Brazil	6	0.1164	<0.001	LD
Argentina	4	0.0037	ns	LE
Israel and Australia	6	0.2134	<0.001	LD
Israel	4	0.0052	ns	LE

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

geographic substructuring seems to be the most obvious explanation for the observed LD, contribution of other factors, such as genetic drift, non-random mating, selection and infrequent recombination, cannot be entirely discarded.

To further support the notion of geographic substructuring, the genetic differentiation between groups of samples originating from five geographic regions/countries (North America, Brazil, Argentina, Israel and Australia) was tested using the exact G-test (Raymond and Rousset, 1995; Rousset, 2008). This test proved to be highly significant and the null hypothesis that samples originate from the same population had to be rejected. In addition, a test of differentiation between all pairs of samples showed that no sample pair can be considered to pertain to a single population (data not shown). Finally, we quantified the genetic differentiation (F_{ST}) of the total study group over all loci by analysis of variance (Θ) and Nei's estimation of heterozygosity (G_{ST}') (Weir and Cockerham, 1984; Nei, 1987). A very large value of 0.312 (95% confidence interval: 0.227–0.395) was obtained for Θ (SE = 0.044). In contrast to Θ, G_{ST}' does not correct sample size differences, which might explain its slightly lower value of 0.296. The estimated value for Θ suggests that a high parasite genetic diversity of about 30% has to be ascribed to genetic differences between the parasite samples from the different geographic regions.

As shown in Fig. 1, a dendrogram constructed to visualize similarity of MLGs between reference isolates provided further evidence for the notion of geographic isolation, as isolates originating from Argentina, Brazil, Israel, Australia and from North America (Mexico and USA) respectively, clustered together. Thus, *B. bovis* populations are clearly structured between the studied countries and/or continents. However, with the exception of isolates B4P and B6P, a fine clustering of regional isolates was not observed (e.g. between B3P and B5P, and S2P and M1A, respectively). Generation of trees based on different similarity/distance coefficiency matrices provided evidence for a lower stability of the branch of isolates from Argentina. This

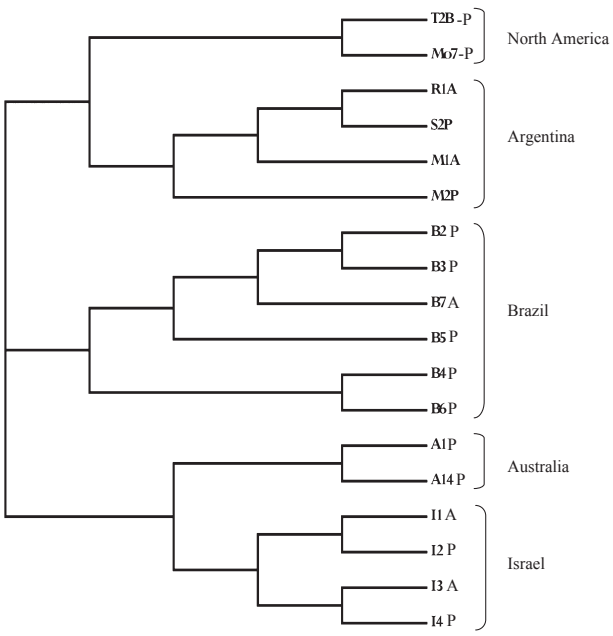

Fig. 1. Dendrogram of pairwise similarity of *B. bovis* isolates. A, attenuated strains; P, pathogenic strains.

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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