

Extensive Genotypic Diversity in a Recombining Population of the Apicomplexan Parasite *Theileria parva*

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We evaluated sexual recombination in the apicomplexan parasite *Theileria parva* using genome-wide marker analysis of haploid sporozoite populations obtained from infected *Rhipicephalus appendiculatus* ticks. Analysis of 231 parasite clones derived by in vitro infection of bovine lymphocytes revealed 48 distinct combinations of 64 polymorphic marker loci. One genotype accounted for more than 75% of the clones, and the population was highly inbred with respect to this. The occurrence of frequent recombination was evident from reassortment of contiguous markers in blocks, with some recombination occurring within blocks. Analysis of four polymorphic loci encoding antigens targeted by protective cytotoxic-T-lymphocyte responses confirmed that these loci reassort, both within and between chromosomes, suggesting that recombination may influence immune recognition. Marker analysis of a panel of 142 clones derived from the population after an additional passage through a calf and the same tick colony revealed 18 genotypes, with the original dominant genotype accounting for 75% of the population and a higher level of inbreeding with respect to it in the remaining clones. Selected marker analysis of genomic DNA from these stabilates and the two preceding generations of the isolate, each derived from distinct tick colonies, revealed shifts in population structure with each generation, suggesting that the tick vector may impose nonrandom selective pressure on the parasite.

Sexual reproduction is believed to have evolved to create genetic variation in the face of selective pressure; in the absence of selection, the asexual individual has twice the fitness of that reproducing sexually (16). Apicomplexan parasites alternate between asexual and sexual development in vertebrate and/or invertebrate hosts. The principal driver for sex in these parasites must derive from the host and is likely to relate to resistance mechanisms. *Theileria parva* is an apicomplexan that infects and transforms lymphocytes of cattle and African buffalo. Transmitted by *Rhipicephalus appendiculatus* ticks, the parasite causes a severe lymphoproliferative disease of cattle in eastern, central, and southern Africa. The parasite has a typical apicomplexan life cycle that is predominantly asexual, with only a brief diploid phase in the tick (19). Infective sporozoites are inoculated by the tick and rapidly invade host lymphocytes, where they access the cytosol and differentiate to multinucleate schizonts. This event is associated with transformation of the infected cell to a state of uncontrolled proliferation (3). Clinical disease arises from invasion of lymphoid and nonlymphoid tissues by parasitized cells and is often fatal (13). In a proportion of infected cells schizonts undergo further differentiation to merozoites, which, upon rupture of the cell, invade erythrocytes and develop into piroplasms, the infective stage for

ticks. Recovered animals are almost invariably long-term carriers of this stage of the parasite (15). When ingested by a feeding tick, piroplasms give rise to gametes, which undergo syngamy in the gut to form diploid zygotes. The latter invade gut epithelial cells and undergo further development to produce motile kinetes. There is evidence that this process entails a meiotic reduction division involving interchromosomal cross-over events (6, 23, 24). Kinetes migrate to the salivary gland and invade specialized cells in type III acini, where they undergo further division to form cattle-infective sporozoites.

Infection with *T. parva* is detrimental to the survival of *R. appendiculatus* ticks (31) and, although little is known of tick immune mechanisms, these are likely to mimic those seen in other arthropods. For example, *Anopheles* mosquitoes deploy a number of defensive mechanisms that compromise survival of malaria parasites, including oxidative metabolites in the gut lumen and hemocyte activity (4, 5, 11). Considerably more information is available regarding the bovine immune response to *T. parva*. Recovered cattle are solidly immune to homologous challenge, with immunity being mediated by major histocompatibility complex (MHC) class I-restricted parasite-specific CD8⁺ cytotoxic T lymphocytes (CTL) (18, 21). Studies in populations of cattle have revealed that the CTL response is almost invariably restricted by class I products of only one MHC haplotype (29). Where appropriate markers are available, restriction can often be resolved to a single locus. This suggests the response is tightly focused on only a small number of peptide-MHC combinations.

Field populations of *T. parva* are antigenically heteroge-

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neous (12, 20), and only limited cross-protection is observed between strains (12, 27). Features of cross-protection have been well defined in the context of the Muguga and Marikebuni isolates of the parasite. The former has been passaged extensively and is relatively homogeneous, whereas the latter is heterogeneous at antigenic and molecular levels, having been isolated relatively recently (1, 20). Although all cattle immunized with Marikebuni generate CTL that recognize both parasite populations, Marikebuni-specific CTL responses are observed in only a proportion of Muguga-immunized cattle (28). However, CTL in these animals recognize only a subset of the parasite strains present in Marikebuni, and the composition of this subset varies from animal to animal (8). These observations are consistent with the existence of several polymorphic epitopes, each of which is shared between Muguga and distinct components of the Marikebuni stock. Which of these is targeted by the CTL response appears to be influenced by both host MHC and parasite genotypes. For example, CTL restricted by the African KN104 MHC class I specificity target a shared epitope when induced by infection with a Marikebuni clone but recognize a nonconserved epitope when provoked by Muguga (28). This suggests that the nonconserved determinant in this system is dominant and, when present, in some way constrains induction of cross-reactive CTL. When considered in the context of the tight focus of the CTL response to individual parasites, such immunodominance may provide the parasite with an opportunity to evade the response through sexual recombination. Hence, reassortment of alleles present in a mixed infection ingested by a tick could result in progeny in which the determinant targeted by the response is absent.

Although evidence for genetic crossover of *T. parva* in the tick has been obtained by tracking the segregation of several polymorphic sequences (23), the extent of recombination across the genome has not been determined. In particular, no information is available on the reassortment of CTL determinants. Two recent developments have now made possible an elucidation of these issues. First, the availability of the *T. parva* genome sequence has allowed assembly of a genome-wide panel of satellite markers for high-resolution genotyping of parasite populations. Second, a number of parasite antigens recognized by bovine CTL have been identified (9) and located within the genome. We have exploited these developments to undertake a broad genotypic analysis of a recombining population of *T. parva* before and after transmission through a naive calf and the tick vector. We provide evidence for considerable levels of recombination in the parasite during mating and show that this results in reassortment of CTL determinants during passage through the tick. We also provide evidence for the selection of parasite variants in the tick vector.

MATERIALS AND METHODS

Parasite populations. The study focused on the Marikebuni stock of *T. parva*, which was isolated in 1981 from adult ticks collected in Kilifi District, Kenya (20). The stock underwent three calf-tick passages at the International Laboratory for Research on Animal Diseases (ILRAD) in Nairobi to yield stabilate IL3014 (Table 1). *R. appendiculatus* ticks fed on a calf infected with IL3014 at the National Veterinary Research Centre (NVRC) in Kenya were transported to the Centre for Tropical Veterinary Medicine (CTVM) in Edinburgh and used to prepare stabilate 70 (St70). The freshly prepared stabilate was used to infect a male Friesian calf and passaged through ticks from the CTVM colony to yield

TABLE 1. Genealogy of *T. parva* Marikebuni stabilates used in this study

Passage	Stabilate	<i>R. appendiculatus</i> colony
1	IL1581	ILRAD
2	IL2245	ILRAD
3	IL3014	ILRAD
4	CTVM St70	NVRC
5	CTVM St72	CTVM
6	CTVM St96	CTVM

St72. The latter was further passaged through an additional Friesian calf and CTVM ticks to generate St96.

Animal infections. Calves were infected by subcutaneous inoculation of 1.5 to 2.5 tick equivalents of stabilate behind the right parotid lymph node. The progress of the infection was monitored by evaluation of the rectal temperature on a daily basis from day 5 and microscopic examination of needle aspirates drawn from the draining lymph node on alternate days after the first manifestation of fever for the presence of *T. parva* schizonts. Approximately 1,000 *R. appendiculatus* nymphs enclosed in cloth bags were applied to the ears of each calf on days 9 and 11 postinfection. Engorged nymphs were removed daily and incubated for 4 weeks at 28°C and 85% relative humidity to allow molting. Calves were euthanized on day 17 of infection. Molted adult ticks were prefed on rabbits for 4 days to stimulate sporogony and surface sterilized by sequential rinses in 5% chlorhexidine, 70% ethanol, and antibiotics. A sample was removed prior to sterilization for assessment of the prevalence and abundance of infection in methyl green- and pyronin-stained salivary gland smears (30). Stabilates were prepared by trituration of sterilized ticks essentially as described by Brown (2).

In vitro infection and cloning. Peripheral blood mononuclear cells were isolated from defibrinated jugular venous blood by flotation on Ficoll-Paque as described previously (7). Cells (10^7) were resuspended in 1 ml of RPMI 1640 medium containing 2 mM glutamine, 5×10^{-5} M 2 mercaptoethanol, 100 IU of penicillin/ml, and 100 µg of streptomycin/ml and supplemented with 10% fetal calf serum (culture medium) and mixed with an equal volume of St72 or St96 diluted in culture medium to obtain a multiplicity of infection of ~0.4 sporozoites/cell (assuming 5×10^4 sporozoites/infected acinus). The mixture was incubated for 2 h at 37°C with occasional agitation before the addition of 8 ml of medium and centrifugation at $200 \times g$ for 10 min. Infected cells were then resuspended in culture medium at a density of 2.5×10^6 cell/ml, inoculated in a 24-well plate in aliquots of 1 ml/well, and incubated for 48 h at 37°C in a humidified atmosphere of CO₂ and air. Cells were then harvested, assessed for viability by trypan blue exclusion, and suspended at a density of 10^5 viable cells/ml in culture medium. This suspension was used to seed 96-well plates in aliquots of 100 µl/well, with two plates each at 1×10^4 /well, 3×10^3 /well, 1×10^2 /well, 3×10^2 /well, and 1×10^2 /well. Each well then received 5×10^4 irradiated (50 Gy) autologous peripheral blood mononuclear filler cells in 100 µl of culture medium supplemented with 50% conditioned medium derived from established *T. parva*-infected lymphoblast cultures. Plates were incubated for 2 to 3 weeks at 37°C in a humidified atmosphere of CO₂ in air and screened for the presence of single clones. One-third of each positive well was then harvested for preliminary marker analysis by PCR, and the residual culture was replenished with fresh medium. Unique clones were expanded for full genotyping as required and stored as live stabilates under liquid nitrogen.

Polymorphic markers and genotyping. (i) **Satellite markers.** The satellite markers used here were those described by Oura et al. (26) supplemented with an additional 13 polymorphic markers that had previously been excluded due to cross-reaction with other *Theileria* species. The additional markers were chosen on the basis of their location on the four chromosomes and are outlined in Table 2.

(ii) **Size polymorphisms.** The satellite marker set was further enhanced by inclusion of PCR primers that amplify polymorphic regions of four genes and/or open reading frames of *T. parva* and give rise to amplicon size polymorphisms that distinguish *T. parva* Muguga clone 3308 and *T. parva* Marikebuni clone 3219 as outlined in Table 3.

(iii) **PCR and allocation of allele designations.** PCR conditions were essentially as described by Oura et al. (26), except that the final primer concentration was 1 pM, the PCR buffer was custom-made and purchased from ABgene (United Kingdom), and 40 amplification cycles were used. Satellite PCR products were separated in 2% Metasieve agarose (Flowgen, United Kingdom) gels,

TABLE 2. Additional satellite loci employed in the study, with associated primer sequences^a

Satellite	Chromosome	Sequence		Amplicon size (bp)
		Forward	Reverse	
MS47	1	GTCACAAGGGAAATCATGTCAC	GAGCCTTGAGTAGGTCTAAATTG	398
MS48	1	CTACTTCTGGATCAGGTGTGGTGG	GATTGAGACGATCCCGGTAGTCCT	223
MS49	1	CACACCGAACTTTGATCTCAC	CACAACGGAGATTCATCTAACA	463
MS50	2	AGTTTGTGACCCAACCTTACC	GACGCTGTATCGCATAAATCAC	391
MS51	3	CCACCAACTCACTTTTATCC	GGCAGAAAACCTAACAACAAAC	161
MS52	3	GACTATAGAAGGAAGTGTCCA	ATAGGATTATCCGGTACTTGC	220
MS53	3	GGCGATGAAAATATCAGGT	ACGCCAGCACTTTATTCA	208
MS54	3	GAGTCGTACATTTTCTCAAGG	CTAGTCTCTCCCCTCAGAGTC	348
MS55	3	CGCCTCAATACTCCTAACAC	TGGGCTTAGTCCAGTGTAT	243
MS56	3	AACCCTCTCTTTCAACTCCTA	GATCACGACTCAAGTAACTGC	173
MS57	4	CATGCACACCGTAGGTATATT	AGGTACCACACACACTTTC	118
MS58	4	CGTATCGAACACACAGTTACAC	CCACACACAAATATCTACCACT	300
MS59	4	TCAGATTCCCCAGTATTTC	CAGAATCTCCAAGTCAATTCTC	111

^a The amplicon sizes shown are those found in the *T. parva* Muguga clone 3308 (www. tigr.org).

which were stained with ethidium bromide and visualized and photographed using a UV light box. The amplicon size at each marker locus was recorded and assigned a serial alphabetical code on the basis of the order in which it was first observed during the course of the analysis. Because the marker panel was first evaluated on a clone of the Muguga stock of *T. parva* (Mu273), alleles occurring at all loci in that population were designated A.

RESULTS

Expanded polymorphic marker set. The expanded marker set comprised 64 individual primer sets that discriminate 16, 10, 23, and 17 loci on chromosomes 1, 2, 3, and 4, respectively. When evaluated in St72, the degree of polymorphism was observed to vary among the markers, with the majority discriminating two to three alleles and a small proportion revealing greater numbers. The most discriminatory satellite was MS14, of which eight alleles were present in the stabilate.

The markers were used to genotype a panel of 231 cloned lines derived from St72 in separate cloning experiments. The majority ($n = 57$) of the markers were discriminatory of multiple infections during the cloning process (data not shown), and only those lines yielding a single allele at all 64 loci were included in the analysis to eliminate artifacts associated with PCR bias and template switching (14). A total of 48 genotypes were found in the panel, with one (72-01) accounting for 175 (75.75%) of the clones. Although three additional genotypes were represented by three to four replicates, all of the remainder were present as singletons. Examination of the genotypes at the level of individual chromosomes revealed 22 variants of chromosome 1, 31 variants of chromosome 2, 32 variants of chromosome 3, and 21 variants of chromosome 4. Some chromosomal variants were common to sev-

eral genotypes. Hence, 26 of the genotypes shared chromosomal variants with the 72-01 clone, with 7 sharing three variants, 5 sharing two variants and 14 sharing a single variant. Variants of individual chromosomes differed in their relatedness, with some diverging from the 72-01 genotype by only a single marker and others being heterologous at multiple loci. Where satellite markers were present in close proximity on a given chromosome, it was evident that they reassorted in blocks (Fig. 1), with individual blocks being common to several genotypes. The distribution of these marker blocks among the genotypes was consistent with the occurrence of recombination within the population.

St72 was subjected to consecutive passages through a calf and the CTVM *R. appendiculatus* colony, as described above, to generate St96. A panel of 32 cloned parasitized lines was established by limiting dilution cloning of lymph node aspirates taken during the peak of the schizont parasitosis that arose in the animal. Analysis of the panel using a restricted marker set indicated that 24 of the clones (75%) carried the 72-01 genotype, with each of the remaining lines having distinct marker profiles.

Analysis of 142 cloned lines derived from St96 by in vitro infection and cloning revealed a total of 18 genotypes, with the 72-01 genotype accounting for 122 (85.92%) of the clones. However, 19 of an additional 21 clones derived from the stabilate that were only partially typed before being lost were distinct from 72-01, indicating that its frequency within the population is 74.8%. All of the remaining genotypes were distinct from those found in St72. We determined that 6 of 8 variants of chromosome 1 found in St96 genotypes were novel,

TABLE 3. Size-polymorphic loci examined in the study, with associated primer sequences^a

Gene	Chromosome	Sequence		Amplicon size (bp)
		Forward	Reverse	
TP01_1233	1	GCAAAACGTTTACTCTTGACTT	GAAACCTTGGAAATAAAGCTAACG	1,182
TP01_0966	1	AGATTATTTCTTGGATGATGACGA	AGGAGGTGGAGGTTGTATTAAGT	1,573
TP03_0861	3	GTTATCACGATTCTTCCAAGC	AATCAAGGATTAATGTGCAGGA	1044
TP04_0051	4	CCACTGGTTCTTCCGATGTAA	GTTGTCCAGAACCATCAGCA	792

^a The amplicon sizes shown are those found in the *T. parva* Muguga clone 3308 (www. tigr.org). Gene nomenclature is as used on www.tigr.org.

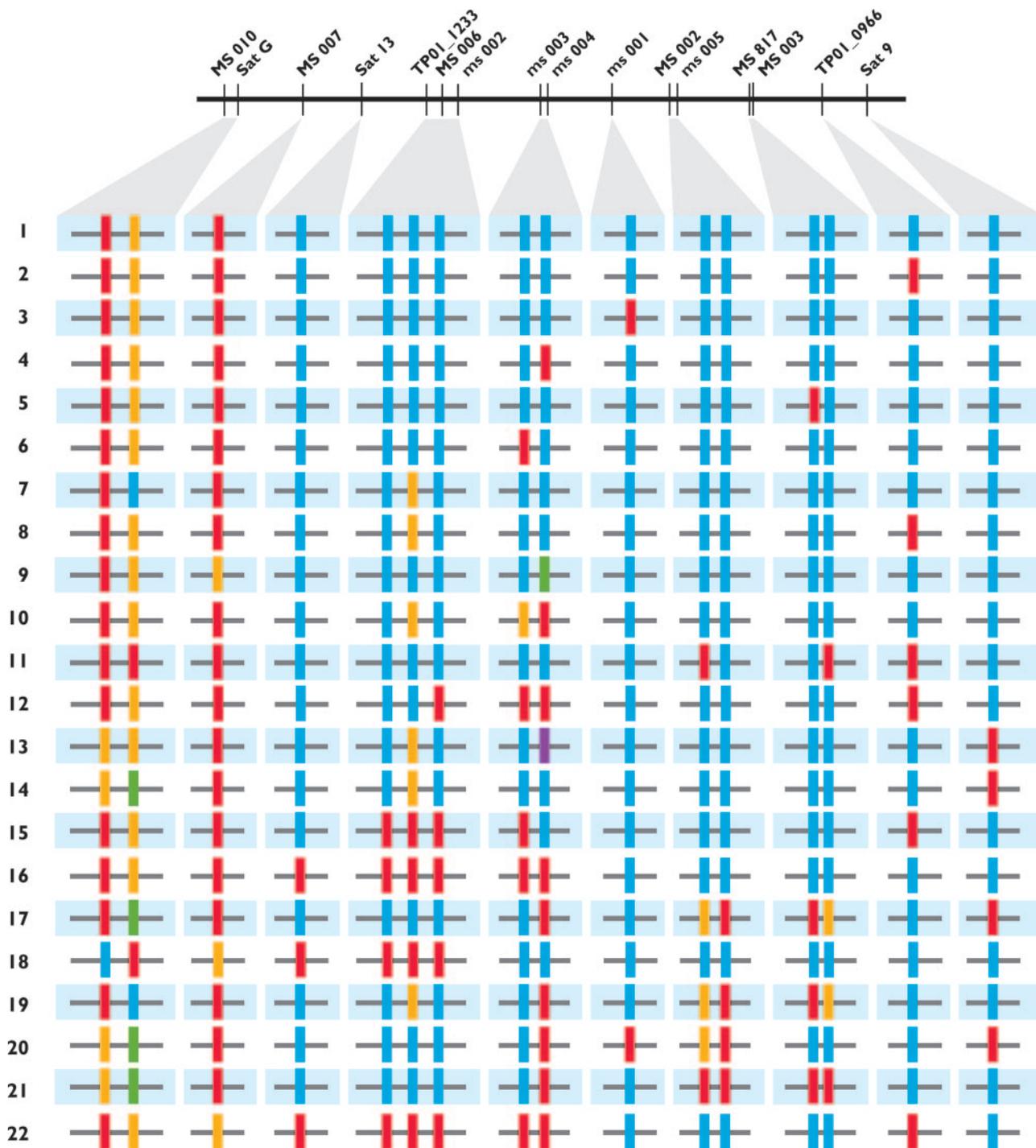


FIG. 1. Schematic representation of the distribution of marker alleles in the 22 variants of *T. parva* chromosome 1 observed in St72. The 72-01 variant is that shown in row 1. Alphabetical codes at each locus have been assigned colors for illustrative purposes as follows: A, blue; B, red; C, yellow; D, green; E, magenta. Contiguous markers are seen to reassort in blocks, with individual blocks also showing evidence of recombination.

as were 9 of 11 chromosome 2 variants, 7 of 9 chromosome 3 variants, and 8 of 9 chromosome 4 variants.

Population structure. The St72 population was highly inbred with respect to the 72-01 clone, with only 10 of the genotypes sharing <70% of its alleles (Fig. 2). Indeed, even the most extremely divergent genotype retained 45% of the 72-01 al-

leles. Clustering analysis based on a distance matrix generated by the LIAN 3.1 software (www.adenine.biz.fh-weihenstephan.de/lian/) yielded eight distinct clusters (Fig. 3A). Five of these were made up of relatively closely related genotypes and constituted a supergroup clustering around the 72-01 genotype. The remaining clusters comprised more distantly related geno-

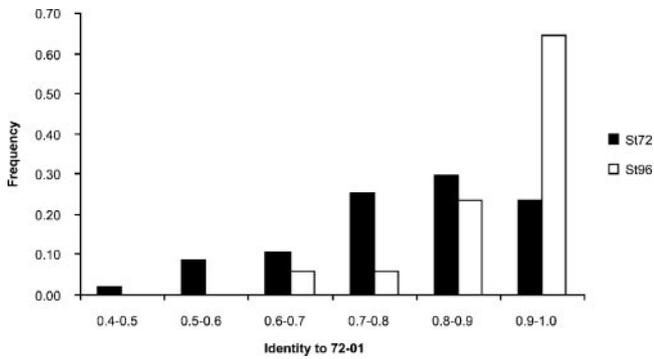


FIG. 2. Inbreeding in St72 and St96 with respect to the 72-01 genotype. Frequencies are provided for genotypes sharing different levels of identity with the clone.

types. Five of the genotypes could not be assigned to clusters. A greater degree of inbreeding was observed in St96, with only 1 of the 18 genotypes showing less than 70% homogeneity with the 72-01 genotype and 9 genotypes sharing more than 90% of

its alleles. This was reflected in the cluster analysis (Fig. 3B), which revealed only four clusters and six nonclustering genotypes. Given the evidence for recombination and the preponderance of the 72-01 genotype in each, both stabilate populations can be considered to have an epidemic population structure, as defined by Maynard-Smith et al. (17).

Linkage equilibrium, as inferred by the statistical independence of alleles at all loci, was evaluated for each stabilate by using the LIAN 3.1 software (10). This program conducts pairwise comparisons of haplotypes and computes the number of loci at which each pair differs. A variance, V_D , is calculated from the distribution of mismatch values and compared to V_e , the variance expected for linkage equilibrium. A standardized index of association (I_A) is derived from the ratio of V_D to V_e and is zero for linkage equilibrium, with values significantly greater than zero reflecting linkage disequilibrium. When conducted on both stabilates at the level of the entire population, this analysis yielded I_A values that were greater than zero, a finding consistent with the occurrence of linkage disequilibrium and low levels of recombination (Table 4). Greater than zero (albeit lower) I_A values were

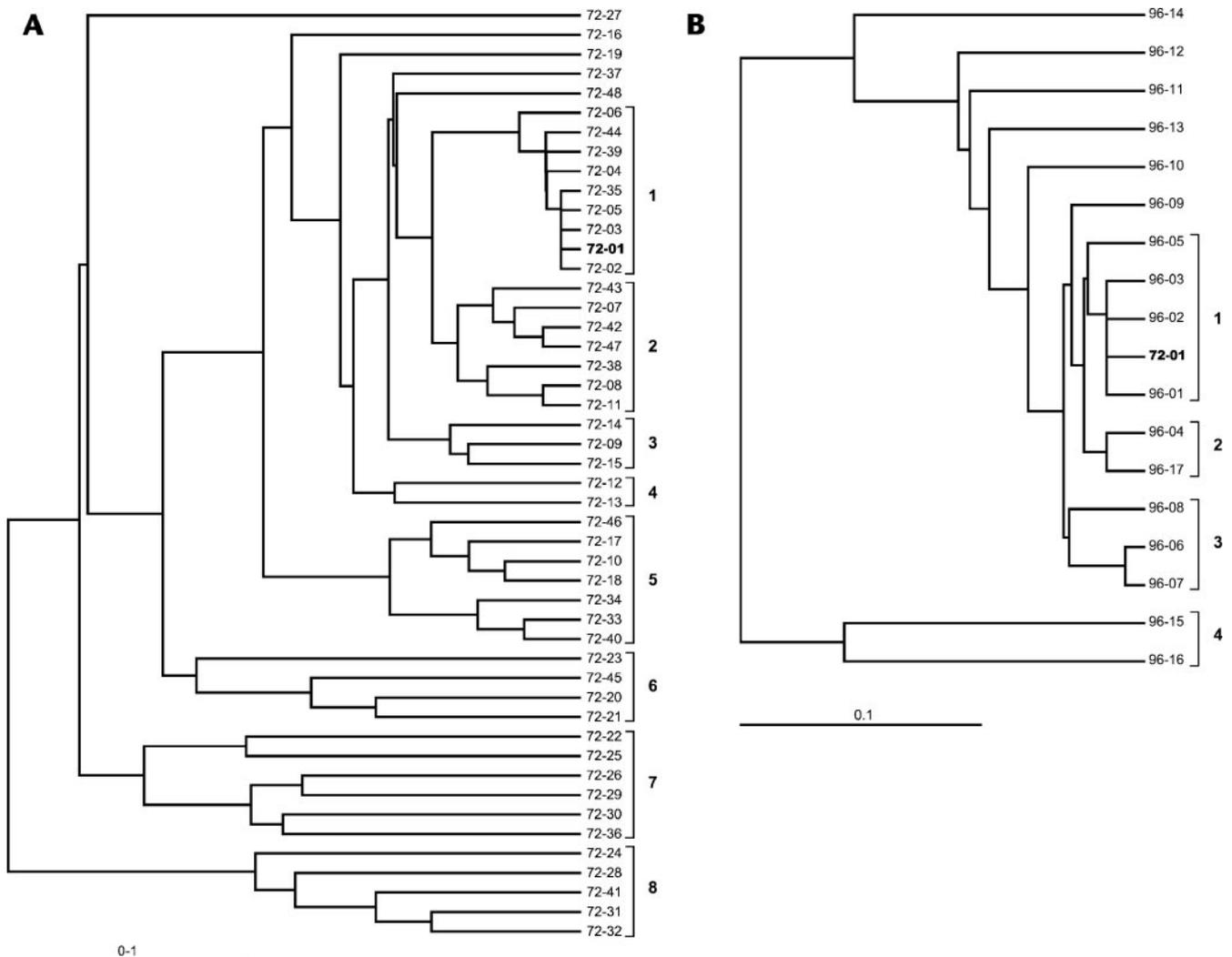


FIG. 3. Cluster analysis of genotypes in St72 (A) and St96 (B) based on pairwise distance matrices generated by the LIAN 3.1 software. Major clusters are indicated, and the position of the 72-01 genotype is highlighted in boldface text.

TABLE 4. Linkage analysis of genotypes in two *T. parva* Marikebuni stabilates using LIAN 3.1 software^a

Parameter	St72		St96	
	Clones	Genotypes	Clones	Genotypes
Total no.	231	48	142	18
<i>D</i>	0.081 (0.005)	0.317 (0.017)	0.029 (0.004)	0.159 (0.017)
<i>I_A</i>	0.241	0.104	0.158	0.0963

^a www.adenine.biz.fh-weihenstephan.de/lian/. *D*, mean genetic distance between loci; *I_A*, standardized index of association. Standard errors are indicated in parentheses.

also returned when identical clones were treated as one and the populations were analyzed at the level of individual genotypes.

Segregation of CTL antigens. To evaluate whether reassortment occurs between loci encoding antigens targeted by the host CTL response during recombination, three antigens, designated Tp1, Tp4, and Tp5 (9), were chosen on the basis of PCR-restriction fragment length polymorphism between parasite clones. A fourth antigen, Tp9 (N. D. MacHugh and S. P. Graham, unpublished data), which exhibits size polymorphism (data not shown), was also examined. The Tp1 and Tp4 loci lie on chromosome 3 of the parasite, separated by approximately 1.36 Mb, whereas those of Tp5 and Tp9 lie on chromosome 2 interspersed by approximately 0.28 Mb (9; N. D. MacHugh and S. P. Graham, unpublished data). PCR-restriction fragment length polymorphism analysis of clones derived from St72, using the MseI, MspI, and AluI restriction enzymes, distinguished two alleles at each of the Tp1, Tp4, and Tp5 loci, while two distinct amplicon sizes were observed by PCR amplification of Tp9 (data not shown). Analysis of 28 clones derived from the stabilate by in vitro infection and cloning revealed six allele combinations at the four loci (Table 5), with that carried by the 72-01 genotype accounting for 16 of the clones. When the analysis was restricted to the two antigen loci on each chromosome, three of the four possible allele combinations were observed among the clones in each case. This provides evidence for the reassortment of antigenic loci between and within chromosomes.

Stability of population structure on passage. Material was available at the CTVM from St70, the progenitor of St72. In vitro infection and cloning experiments with this stabilate were hampered by a low level of infectivity and yielded only five clones, all of which were distinct from those obtained from St72. Although three of these clones showed more than 60% homology with the 72-01 genotype, the remaining two shared less than 30% of its alleles. In vitro generation of a representative panel of parasite clones from ILRAD stabilate IL3014, the progenitor of St70, was also unsuccessful, due to the presence of contaminating molds. However, three clones derived previously from IL3014-infected cell lines (8) were available for satellite analysis. None of these clones showed greater than 16% homology with the 72-01 genotype. Two additional IL3014 clones, derived independently from separate in vitro limiting dilution infections (22, 28), were also available, in the form of sporozoite stabilates (IL3219 and IL3263). Satellite analysis of these clones revealed identical genotypes that shared only 30% homology with 72-01. Despite the lack of representative numbers of clones from these stabilates, it was possible to evaluate their overall composition by satellite anal-

TABLE 5. Antigenic variants and their frequencies among 28 clones derived from St72 as detected by PCR analysis of four biallelic loci^a

Antigenic variant	Chromosome 2		Chromosome 3		Frequency
	Tp5	Tp9	Tp1	Tp4	
1	A	A	A	A	0.14
2	A	A	A	B	0.14
3	A	B	A	A	0.04
4	A	B	A	B	0.57
5	A	B	B	B	0.04
6	B	B	B	B	0.07

^a Alleles are designated A or B. Genotype 72-01 corresponds to variant 4.

ysis of the entire populations using selected markers (Fig. 4). Whole stabilate satellite analysis confirmed that St72 and St96 are dominated by clones carrying the 72-01 allele for each of the markers used. In contrast, IL3014 is dominated by a distinct allele at each of the loci examined. St70 is in most cases intermediate, with both the 72-01 and the IL3014 allele being present; for two of the markers used, evidence for additional heterogeneity is present. These observations suggest two significant shifts in the population structure of the Marikebuni isolate during passage from IL3014 to St70 and from St70 to St72. On the contrary, the overall molecular fingerprint of the isolate remained relatively conserved during passage from St72 to St96.

DISCUSSION

We describe an in-depth multilocus genotyping analysis of *T. parva* populations in two generations of the Marikebuni stock of the parasite obtained by passage through the CTVM *R. appendiculatus* tick line. Using an expanded panel of satellite and other polymorphic markers based on those described by Oura et al. (26), considerable genotypic variation was observed in the stock, despite only limited diversity at individual loci. Although only two or three alleles were observed at the majority of loci, 48 distinct locus combinations were observed in St72 of the stock, with one, 72-01, being highly dominant. This suggests that the parasite has considerable potential for recombination during the sexual phase of its life cycle. Reassortment of loci within the population was also evident from the distribution of blocks of alleles at contiguous loci among genotypes and apparent recombination within blocks. Further evidence for substantive recombination in *T. parva* arose from analysis of the progeny of St72 after an additional calf-tick passage. Although still dominated by the 72-01 genotype, the daughter St96 incorporated several new variants of each chromosome and contained none of the other genotypes found in St72. In addition, a substantially increased degree of homogeneity with respect to 72-01 alleles was observed in the other genotypes. Two lines of evidence argue that the minor genotypes observed in the analysis were true subpopulations of the stabilate rather than products of satellite hypermutation during the cloning process. Satellite hypermutations in culture would inevitably have manifested as mixed infections and resulted in exclusion from the analysis. Second, identical minor genotypes were observed in separate cloning experiments with both St72 and St96 (data not shown), indicating that they are stable and a true component of the stabilate.

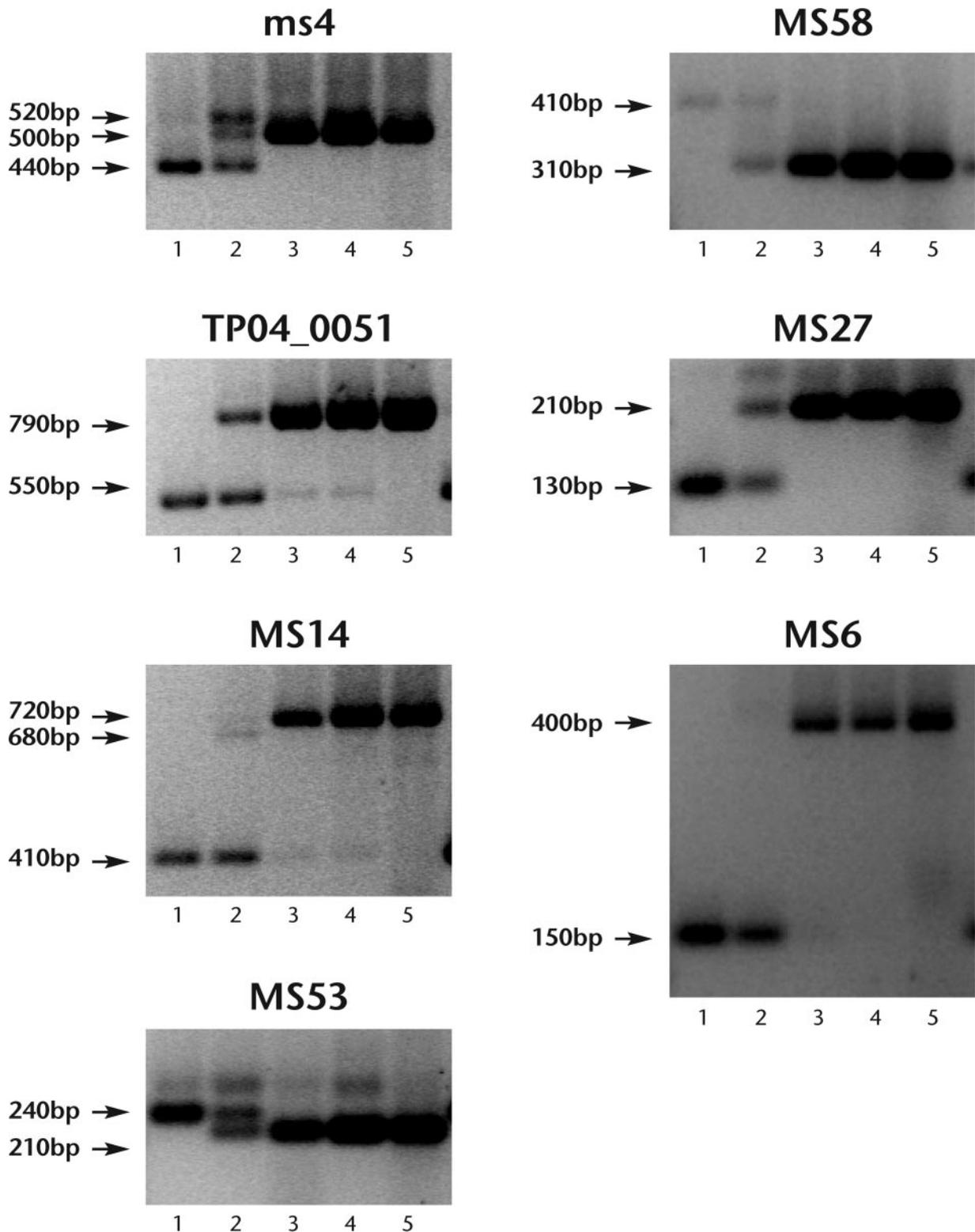


FIG. 4. Marker analysis of whole genomic DNA prepared from four successive generations of the Marikebuni isolate of *T. parva*. Lane 1, IL3014; lane 2, St70; lane 3, St72; lane 4, St96; 5, clone 72-01.

An important consequence of the observed recombination was that it resulted in shuffling of immunological determinants: several distinct combinations of alleles were observed among the St72 genotypes for pairs of loci that encode antigens tar-

geted by the CTL response and collocate to chromosomes 2 and 3, respectively. This confirms that CTL antigen loci reassort during meiotic recombination and suggests that the parasite has potential for immune evasion at the herd level. How-

ever, the impact of such recombination events is unclear in the absence of information on allelic polymorphism among CTL antigens. Indeed, it remains unclear whether the parasite has an evolutionary requirement for immune evasion; it is not yet known whether the CTL response significantly compromises transmission.

In apparent conflict with the observed evidence for frequent recombination in mating *T. parva* populations, a statistical analysis of linkage equilibrium among marker loci using the LIAN 3.1 software yielded low values for mean genetic diversity across the loci (Table 4) and standardized I_A values greater than zero, whether the population was examined as a whole (i.e., incorporating data from every clone) or when the analysis was confined only to genotypes. This implies significant linkage disequilibrium between the satellite loci, which almost certainly arises from the high levels of inbreeding in the population with respect to the 72-01 genotype, with homogeneity at individual loci masking statistical independence in reassortment.

The dominance of the 72-01 genotype in the parasite stabilates examined in the study provides valuable insights into the biology of *T. parva*. The observed frequency of the genotype in St72 indicates that it was carried, on average, by 75% of the sporozoites in each infected tick that gave rise to the stabilate. Given the nature of the parasite life cycle, this could have been achieved only if 75% of kinetes generated in each tick carried the genotype, which in turn requires that 75% of progenitor zygotes were homozygous for the genotype. Assuming equal proportions of male and female gametes (32), this would necessitate a frequency of at least $0.87 (\sqrt{0.75})$ for 72-01 gametes in the tick gut prior to syngamy. In the light of the high levels of attrition observed for *T. parva* piroplasms and gametes after ingestion by the tick (25), these figures suggest either preferential survival of 72-01 gametes from a more heterogeneous population or some form of selection for 72-01 piroplasms in the calf of origin. The latter alternative cannot be ruled out but seems unlikely, given that the calf was of United Kingdom origin and *T. parva* naive. Furthermore, the parasite population that arose in a naive calf challenged with St72 in the present study (see above) showed no evidence of selection for the 72-01 genotype, with a frequency of 75% being retained in parasitized cells collected during the schizont parasitosis. On the other hand, the argument for preferential survival of this genotype in the tick is supported by its frequency in the stabilate derived from ticks fed on this animal. Assuming equal levels of merogony among the clones, a maximum of 75% of piroplasms ingested by the ticks could have carried the genotype, given its proportion in the schizont population. In the absence of selection, this would yield a frequency of only 0.75^2 , or 0.56, for homozygous zygotes, and, consequently, their sporozoite progeny. In contrast, the frequency of the 72-01 genotype in parasite clones derived from St96 after in vitro infection suggests that 75% of its sporozoites carry the genotype. As discussed above, this would require that it account for 87% of both male and female progenitor gametes. Such an increase in frequency could only have occurred through selection against other genotypes prior to syngamy.

Some support for this possibility emerges from the marker analysis of four generations of the Marikebuni stock described above. Although St72 and its daughter St96 showed essentially

identical profiles (dominated by the 72-01 allele) with each of the markers used, that of the progenitor St70 was clearly distinct. St70 was derived from the NVRC *R. appendiculatus* colony (Table 1), whereas both St72 and St96 were generated by using the CTVM colony. Laboratory tick colonies are generally maintained by using limited numbers of breeding individuals and, as a result, are inevitably inbred. An attractive explanation for the dramatic expansion of the 72-01 profile during transmission of St70 and St72 and its maintenance in St96 is substantial selection for that genotype by the CTVM tick line. If true, this would imply variation between tick populations in the specificity of selection, with CTVM and NVRC tick lines showing distinct preferences. Consistent with this notion, the IL3014 stabilate, which is the product of three consecutive passages through the ILRI tick colony, is genotypically distinct from its daughter St70 and the subsequent stabilates. Furthermore, only a single allele was observed for several of the markers used to characterize IL3014, which suggests that it, too, is dominated by a single genotype. This might also be concluded from the emergence of an identical genotype from this stabilate in two separate cloning experiments.

Taken together, these observations suggest that different inbred tick populations may favor distinct components of the Marikebuni stock and, further, that repeated passage of the stock through individual tick lines is associated with increasing homogeneity with respect to the favored population. The latter issue probably has little relevance to the field situation but has substantial implications for the maintenance of stabilates for large-scale infection and treatment immunization, which depends on heterogeneity for breadth of cover (27). However, variation among tick populations in the specificity of selection for *T. parva* parasites has major consequences for the population structure of the parasite in the field, since it identifies tick population heterogeneity as a major determinant of parasite diversity. In this regard, it is useful to consider the potential selection imposed by the tick. A female *R. appendiculatus* nymph ingests approximately 80 μl of blood during the course of feeding, of which roughly 80% is ingested during the final engorgement phase of feeding. On the basis of a bovine erythrocyte count of $5 \times 10^6/\mu\text{l}$ and an average acute piroplasm parasitemia of 5%, this equates to a potential uptake of 1.6×10^7 piroplasms during engorgement. Even highly abundant tick infections are of the order of only 10^2 sporoblasts (25), which is consistent with parasite losses of 5 orders of magnitude during differentiation in the tick. A selection process of this stringency, if nonrandom and variable between ticks, demands substantial potential for diversity in *T. parva* and may provide some explanation for the considerable genotypic plasticity observed in these studies. It may also account for the tight focus of the bovine CTL response to individual *T. parva* strains, which would be expected to favor carrier infections with mixed genotypes and encourage genetic recombination.

The identification of a number of *T. parva* antigens targeted by bovine CTL has paved the way for development of a subunit vaccine against the parasite. The extent of antigenic diversity in field populations of *T. parva* will be an important determining factor in the efficacy of such a vaccine. However, the observed potential of the parasite for adaptive response through rapid selection of genotypes with favorable gene combinations will

undoubtedly present additional challenges with regard to its durability.

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REFERENCES

- Bishop, R. P., B. K. Sohanpal, B. A. Allsopp, P. R. Spooner, T. T. Dolan, and S. P. Morzaria. 1993. Detection of polymorphisms among *Theileria parva* stocks using repetitive, telomeric, and ribosomal DNA probes and antischizont monoclonal antibodies. *Parasitology* **107**:19–31.
- Brown, C. G. D. 1987. Theileriidae, p. 230–253. In A. E. R. Taylor and J. R. Baker (ed.), *Methods for parasite cultivation*. Academic Press, Inc., New York, N.Y.
- Brown, C. G. D., D. A. Stagg, R. E. Purnell, G. K. Kanhai, and R. C. Payne. 1973. Infection and transformation of bovine lymphoid cell in vitro by infective particles of *Theileria parva*. *Nature* **245**:101–103.
- Dimopoulos, G., H. M. Muller, and F. C. Kafatos. 1999. How does *Anopheles gambiae* kill malaria parasites? *Parasitologia* **41**:169–175.
- Dimopoulos, G., D. Seelye, A. Wolf, and F. C. Kafatos. 1998. Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *EMBO J.* **17**:6115–6123.
- Gauer, M., U. Mackenstedt, H. Mehlhorn, E. Schein, F. Zapf, E. Njenga, A. Young, and S. Morzaria. 1995. DNA measurement and ploidy determination of developmental stages in the life cycles of *Theileria annulata* and *T. parva*. *Parasitol. Res.* **81**:565–574.
- Goddeeris, B. M., and W. I. Morrison. 1988. Technique for the generation, cloning and characterization of bovine cytotoxic T cells specific for the protozoan *Theileria parva*. *J. Tissue. Cult. Methods* **11**:101–110.
- Goddeeris, B. M., W. I. Morrison, P. G. Toye, and R. Bishop. 1990. Strain specificity of bovine *Theileria parva*-specific cytotoxic T cells is determined by the phenotype of the restricting class I MHC. *Immunology* **69**:38–44.
- Graham, S. P., R. Pelle, Y. Honda, D. M. Mwangi, N. J. Tonukari, M. Yamage, E. J. Glew, E. P. De Villiers, T. Shah, R. Bishop, E. Abuya, E. Awino, J. Gachanja, A. E. Luyai, F. Mbwika, A. M. Muthiani, D. M. Ndegwa, M. Njahira, J. K. Nyanjui, F. O. Onono, J. Osaso, R. M. Saya, C. Wildmann, C. M. Fraser, I. Maudlin, M. J. Gardner, S. P. Morzaria, S. Loosmore, S. C. Gilbert, J. C. Audonnet, B. P. van der, V. Nene, and E. L. Taracha. 2006. *Theileria parva* candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **103**:3288–3291.
- Haubold, B., and R. R. Hudson. 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. *Linkage Analysis. Bioinformatics* **16**:847–848.
- Hoffmann, J. A. 1995. Innate immunity of insects. *Curr. Opin. Immunol.* **7**:4–10.
- Irvin, A. D., D. A. E. Dobbelaere, B. M. Mwamanchi, T. Minami, P. R. Spooner, and J. G. R. Ocama. 1983. Immunization against East Coast fever: correlation between monoclonal antibody profiles of *Theileria parva* stocks and cross-immunity in vivo. *Res. Vet. Sci.* **35**:341–346.
- Irvin, A. D., and W. I. Morrison. 1987. Immunopathology, immunology and immunoprophylaxis of *Theileria* infections, p. 223–274. In E. J. L. Soulsby (ed.), *Immune responses in parasitic infections: immunology, immunopathology and immunoprophylaxis*, vol. 3. CRC Press, Inc., Boca Raton, Fla.
- Kanagawa, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). *J. Biosci. Bioeng.* **96**:317–323.
- Kariuki, D. P., A. S. Young, S. P. Morzaria, A. C. Lesan, S. K. Mining, P. Omwoyo, J. L. Wafula, and D. H. Molyneux. 1995. *Theileria parva* carrier state in naturally infected and artificially immunized cattle. *Trop. Anim. Health Prod.* **27**:15–25.
- Maynard Smith, J. 1978. *The evolution of sex*. Cambridge University Press, Cambridge, England.
- Maynard Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt. 1993. How clonal are bacteria? *Proc. Natl. Acad. Sci. USA* **90**:4384–4388.
- McKeever, D. J., E. L. N. Taracha, E. A. Innes, N. D. MacHugh, E. Awino, B. M. Goddeeris, and W. I. Morrison. 1994. Adoptive transfer of immunity to *Theileria parva* in the CD8⁺ fraction of responding efferent lymph. *Proc. Natl. Acad. Sci. USA* **91**:1959–1963.
- Mehlhorn, H., and E. Schein. 1984. The piroplasms: life cycle and sexual stages. *Adv. Parasitol.* **23**:37–103.
- Minami, T., P. R. Spooner, A. D. Irvin, J. G. R. Ocama, D. A. E. Dobbelaere, and T. Fujinaga. 1983. Characterisation of stocks of *Theileria parva* by monoclonal antibody profiles. *Res. Vet. Sci.* **35**:334–340.
- Morrison, W. I., B. M. Goddeeris, A. J. Teale, C. M. Grocock, S. J. Kemp, and D. A. Stagg. 1987. Cytotoxic T cells elicited in cattle challenged with *Theileria parva* (Muguga): evidence for restriction by class I MHC determinants and parasite strain specificity. *Parasite Immunol.* **9**:563–578.
- Morzaria, S. P., T. T. Dolan, R. A. I. Norval, R. P. Bishop, and P. R. Spooner. 1995. Generation and characterisation of cloned *Theileria parva* parasites. *Parasitology* **111**:39–49.
- Morzaria, S. P., J. R. Young, P. R. Spooner, T. T. Dolan, and R. P. Bishop. 1993. *Theileria parva*: a restriction map and genetic recombination, p. 67–73. In S. P. Morzaria (ed.), *Genome analysis of protozoan parasites*. International Laboratory for Research on Animal Diseases, Nairobi, Kenya.
- Nene, V., S. Morzaria, and R. Bishop. 1998. Organisation and informational content of the *Theileria parva* genome. *Mol. Biochem. Parasitol.* **95**:1–8.
- Norval, R. A. I., B. D. Perry, and A. S. Young. 1992. The epidemiology of theileriosis in Africa. Academic Press, London, England.
- Oura, C. A. L., D. O. Odongo, G. W. Lubega, P. R. Spooner, A. Tait, and R. P. Bishop. 2003. A panel of microsatellite and minisatellite markers for the characterisation of field isolates of *Theileria parva*. *Int. J. Parasitol.* **33**:1641–1653.
- Radley, D. E., C. G. D. Brown, M. J. Burridge, M. P. Cunningham, I. M. Kirimi, R. E. Purnell, and A. S. Young. 1975. East Coast Fever. 1. Chemoprophylactic immunization of cattle against *Theileria parva* (Muguga) and five *Theileria* strains. *Vet. Parasitol.* **1**:35–41.
- Taracha, E. L. N., B. M. Goddeeris, S. P. Morzaria, and W. I. Morrison. 1995. Parasite strain specificity of precursor cytotoxic T cells in individual animals correlates with cross-protection in cattle challenged with *Theileria parva*. *Infect. Immun.* **63**:1258–1262.
- Taracha, E. L. N., B. M. Goddeeris, A. J. Teale, S. J. Kemp, and W. I. Morrison. 1995. Parasite strain specificity of bovine cytotoxic T-cell responses to *Theileria parva* is determined primarily by immunodominance. *J. Immunol.* **155**:4854–4860.
- Walker, A. R., S. B. McKellar, L. J. Bell, and C. G. D. Brown. 1979. Rapid quantitative assessment of *Theileria* infection in ticks. *Trop. Anim. Health. Prod.* **11**:21–26.
- Watt, D. M., and A. R. Walker. 2000. Pathological effects and reduced survival in *Rhipicephalus appendiculatus* ticks infected with *Theileria parva* protozoa. *Parasitol. Res.* **86**:207–214.
- West, S. A., T. G. Smith, and A. F. Read. 2000. Sex allocation and population structure in apicomplexan (protozoa) parasites. *Proc. Biol. Sci.* **267**:257–263.

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