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Construction of a genetic map for *Theileria parva*: Identification of hotspots of recombination

Frank Katzer [b,1], Regina Lizundia [a,1], Daniel Ngugi [a], Damer Blake [a], Declan McKeever [a,*]

[a] Royal Veterinary College, University of London, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire AL9 7TA, UK
[b] Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian EH26 0PZ, UK

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ABSTRACT

The tick-borne protozoan parasite *Theileria parva* is the causal agent of East Coast Fever (ECF), a severe lymphoproliferative disease of cattle in eastern, central and southern Africa. The life cycle of *T. parva* is predominantly haploid, with a brief diploid stage occurring in the tick vector that involves meiotic recombination. Resolved genetic studies of *T. parva* are currently constrained by the lack of a genome-wide high-definition genetic map of the parasite. We undertook a genetic cross of two cloned isolates of *T. parva* to construct such a map from 35 recombinant progeny, using a genome-wide panel of 79 variable number of tandem repeat markers. Progeny were established by in vitro cloning of cattle lymphocytes after infection with sporozoites prepared from *Rhipicephalus appendiculatus* ticks fed on a calf undergoing a dual infection with the two clonal parental stocks. The genetic map was determined by assigning individual markers to the four chromosome genome, whose physical length is approximately 8309 kilobasepairs (Kb). Segregation analysis of the markers among the progeny revealed a total genetic size of 1683.8 centiMorgans (cM), covering a physical distance of 7737.62 Kb (~93% of the genome). The average genome-wide recombination rate observed for *T. parva* was relatively high, at 0.22 cM Kb^{-1} per meiotic generation. Recombination hot-spots and cold-spots were identified for each of the chromosomes. A panel of 27 loci encoding determinants previously identified as immunorelevant or likely to be under selection were positioned on the linkage map. We believe this to be the first genetic linkage map for *T. parva.* This resource, with the availability of the genome sequence of *T. parva*, will promote improved understanding of the pathogen by facilitating the use of genetic analysis for identification of loci responsible for variable phenotypic traits exhibited by individual parasite stocks.

1. Introduction

Theileria parva is a tick-borne parasite of cattle that infects and transforms bovine lymphocytes, causing a severe lymphoproliferative disease known as East Coast Fever (ECF). Transmitted by the brown ear tick *Rhipicephalus appendiculatus*, the parasite is prevalent in eastern, central and southern Africa. The pathogenesis of ECF arises largely from the invasion of lymphoid and non-lymphoid tissues with proliferating infected lymphoblasts, and susceptible animals normally die within 3 weeks of infection. With an estimated 24 million cattle at risk of infection (Mukhebi et al., 1992) the disease is a major constraint to livestock productivity in the region. The life cycle of *T. parva* is similar to that of other apicomplexa and involves obligate developmental stages in both mammalian and vector hosts (Mehlhorn and Shein, 1984). It is predominantly haploid, with only a brief diploid phase in the tick. Cattle become infected by inoculation of sporozoite forms in tick saliva. Sporozoites invade host lymphocytes and differentiate to multinucleate schizont forms, which transform the infected cell to a state of uncontrolled proliferation. The parasite replicates in synchrony with the dividing lymphocyte and, by associating with the mitotic spindle (Hulliger et al., 1964), ensures transmission of infection to each daughter cell. With the progress of infection, the parasite ceases to divide in a proportion of infected cells and differentiates to uninucleate merozoites. These are released from the dying cell and invade erythrocytes, where they develop into tick-infective piroplasms. Cattle that recover from infection are almost invariably long-term carriers of these forms. Upon ingestion by a subsequent feeding tick, piroplams give rise to macro- and micro-gametes, which undergo syngamy in the gut lumen to yield diploid zygotes. These invade gut epithelial cells and undergo further differentiation, including meiotic division, to form motile kinetes, which migrate to the tick salivary gland. There they undergo further nuclear division, ultimately giving rise to cattle-infective uninucleate sporozoites. We have recently provided evidence for substantial genetic recombination between *T. parva* strains during

* Corresponding author. Tel.: +44 0 1707 666572; fax: +44 0 1707 666298.
E-mail address: dmckeever@rvc.ac.uk (D. McKeever).
[1] Both first authors contributed equally to this work.

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passage through *R. appendiculatus* ticks (Katzer et al., 2006). Cattle can be immunised against *T. parva* by simultaneous inoculation of live sporozoites and long-acting formulations of oxytetracycline, a process known as 'infection and treatment' (Radley et al., 1975). There is strong evidence that the resulting protection is mediated by parasite-specific major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs), which eliminate schizont-infected lymphoblasts (McKeever et al., 1994). The response is highly specific and therefore vulnerable to breakthrough by heterologous strains. Importantly, because it does not engage until the schizont parasitosis is established, the CTL response does not prevent infection, even after challenge with the immunising strain (Morrison et al., 1987). We have recently observed that, although CTLs impose considerable numerical attrition on targeted parasitised lymphoblasts in vivo, a proportion of parasites nonetheless emerge as piroplasms in the erythrocyte compartment. Responses are almost invariably restricted by one or other of the parental MHC haplotypes and generally focus almost entirely on a single antigenic determinant (MacHugh et al., 2009). In addition, the specificity of the response varies with MHC haplotype, suggesting that individual cattle in an outbred herd are likely to target distinct parasite components. We have shown that *T. parva* CTL antigens reassort during recombination in the tick (Katzer et al., 2006) and raised the possibility that this could result in evasion of such a tightly focussed effector response. In the face of immune selection, such an eventuality might be anticipated to result in augmented recombination of CTL antigenic loci over evolutionary time. In both yeast and human genomes, recombination occurs predominantly at specific regions known as hot spots (Petes, 2001; McVean et al., 2004), which can vary considerably in size (from hundreds of bp to tens of megabases (Mb)). A segment of DNA that undergoes recombination at the genome average rate can also be considered a hot spot if it is embedded in a very "cold" region of the genome. Although evidence for genetic crossover of *T. parva* in the tick has been obtained by satellite marker analysis (Katzer et al., 2006) and by tracking the segregation of several polymorphic sequences (Morzaria and Young, 1993), the extent of recombination and the location of hotspots across the genome remain to be determined. The recent availability of the *T. parva* genome sequence (Gardner et al., 2005) facilitated the establishment of a genome-wide panel of satellite markers for high-resolution genotyping of parasite populations. We have exploited these developments to undertake a broad genotypic analysis of a set of recombinant *T. parva* clones and their parents, with the aim of constructing, for the first time, a genetic map of the parasite. We draw on the information provided to evaluate the evidence for selection arising from bovine immunity and from other sources.

2. Materials and methods

2.1. Parasite populations

The study focused on two cloned stocks of *T. parva*, both of which were generated as described by Morzaria et al. (1995). Clone 3308 was derived from the Muguga (3087) stock of *T. parva*, while clone 4210 originated from the Marikebuni (3014) stock. The origins of these stocks have been described previously (Morzaria et al., 1995).

2.2. Animal infections

A male Boran (*Bos indicus*) calf was infected by s.c. inoculation behind the left and right parotid lymph nodes with sub-lethal quantities of sporozoites of both 3308 and 4210 clones. Doses were calculated to provide similar piroplasm parasitaemias for each clone on the basis of previous observations in vivo. The progress of infection was monitored by daily evaluation of rectal temperature from day 5, and microscopic examination of needle aspirates of the draining lymph node for the presence of *T. parva* schizonts on alternate days after the first manifestation of fever. Levels of parasitaemia for each clone were monitored during infection by PCR amplification of DNA extracted from whole blood, using discriminatory satellite markers. Approximately 1000 *R. appendiculatus* nymphs enclosed in cloth bags were applied at regular intervals to each ear of the calf from day 9 p.i. to feed during the piroplasm parasitaemia. Upon detachment, engorged ticks were evaluated for relative levels of infection with each parasite clone by satellite marker analysis of a representative sample. Those that detached on day 21 p.i. produced the most equivalent ratio for the two genotypes, and were chosen for stabilate production. Nymphs were removed after engorgement and incubated for 4 weeks at 28 °C and 85% relative humidity to promote moulting. Moulted infected adult ticks were pre-fed on rabbits for 4 days to stimulate sporogony. They were then removed and surface-sterilized by sequential rinses in 5% chlorhexidine, 70% ethanol and antibiotics, and analysed for parasite infection. The stabilate (CTVM St102) was prepared by trituration of sterilized ticks essentially as described by Brown (1987). All animal experiments were conducted with the formal approval of the institutional animal ethics committees. Standards of care and maintenance for experimental animals were in accordance with government and institutional guidelines.

2.3. In vitro infection and cloning

Peripheral blood mononuclear cells were isolated from defibrinated jugular venous bovine blood by flotation on Ficoll-Paque as described previously (Goddeeris and Morrison, 1988). Cells (10^7) were resuspended in 1 ml of RPMI 1640 medium containing 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% FCS (culture medium) and mixed with an equal volume of CTVM St102 diluted in culture medium, to obtain a multiplicity of infection of one tick equivalent/ml. The suspension was incubated for 2 h at 37 °C with occasional agitation before the addition of 8 ml culture medium and centrifugation at 200 *g* for 10 min. Infected cells were then resuspended in culture medium at a density of 2.5×10^6 cells/ml, dispensed into a 24-well plate in aliquots of 1 ml/well and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 and air. Cells were then harvested, assessed for viability by trypan blue exclusion and suspended in culture medium at a density of 10^5 viable cells/ml. 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, 1×10^3/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 culture medium supplemented with 50% conditioned medium derived from established *T. parva*-infected lymphoblast cultures. Plates were incubated for 2–3 weeks at 37 °C in a humidified atmosphere of 5% CO_2 in air and screened for the presence of single clones. One-third of each positive well was then harvested for the identification of hybrid clones using 24 polymorphic markers for preliminary screening by PCR analysis (Supplementary Table S1) 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.

2.4. Polymorphic markers and genotyping

2.4.1. Satellite markers

The satellite marker panel described by Katzer et al. (2010) based on the original panel of Oura et al. (2003) was used, supple-