



RESEARCH COMMUNICATION

Cloned *Theileria parva* produces lesser infections in ticks compared to uncloned *T. parva* despite similar infections in cattle

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ABSTRACT

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Experimental transmissions of cloned *Theileria parva* in cattle with *Rhipicephalus appendiculatus* ticks were compared to transmissions with uncloned *T. parva* during studies on the potential for genetic recombination during syngamy of *Theileria* to produce antigenic diversity for evasion of bovine immunity. Prevalence and abundance of *T. parva* infection in adult ticks, which resulted from the feeding of nymphs on the calves, were significantly higher in the uncloned compared to the cloned *T. parva*. Development of sporoblasts of *T. parva* in the ticks to produce infective sporozoites was similar. There was no statistically significant difference in the clinical course of infection in cattle between cloned and uncloned *T. parva*. It was concluded that cloned *T. parva* has characteristics that reduce its viability during the tick stages of its life cycle.

Keywords: Clone, East Coast fever, infection, *Rhipicephalus appendiculatus*, *Theileria parva*

Theileria parva is a protozoan parasite of bovids which is transmitted between cattle by the tick *Rhipicephalus appendiculatus* in East, Central and Southern Africa. Infection of cattle causes East Coast fever with a mortality rate that constrains the profitability of cattle rearing (Irvin & Morrison 1987). When piroplasms of *T. parva* in erythrocytes are ingested by the tick they differentiate into microgametes and macrogametes. These undergo syngamy to form zygotes which invade digestive cells. Then a kinete develops from each zygote and this migrates from the gut to the salivary glands (Schein, Warnecke &

Kirmse 1977; Mehlhorn, Schein & Warnecke 1978; Fawcett, Büscher & Doxsey 1982). The kinete develops in *e* cells of the salivary gland by integration with the metabolism of the *e* cells. Few sporoblasts are produced relative to the number of piroplasms ingested and it is thought that *Theileria* is susceptible to digestion by the tick or tick innate immunity (Purnell & Joyner 1968). When the tick next feeds the sporoblast grows and divides to produce thousands of sporozoites. Tick salivation enables sporozoites to enter cattle and infect lymphocytes. There the schizont stage develops and undergoes further nuclear division (Shaw & Young 1995). The infection of the lymphocytes induces them to divide, each daughter cell taking dividing schizonts with it. The final stage in the lymphocytes is the merozoite which develops within the schizont. Merozoites are released into the blood plasma and infect erythrocytes

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in which they are known as piroplasms. Successful immunity, mediated mainly by cytotoxic lymphocytes, may confine infections with potentially fatal doses of sporozoites to ones that produce few clinical signs and very low parasitaemias of piroplasms in which form the *Theileria* may be transmitted to more ticks (McKeever, Taracha, Innes, MacHugh, Awina, Goddeeris, & Morrison 1994).

Existing vaccination procedures using the infection and treatment method manipulate this cycle to produce sporozoites in cryopreserved form which are inoculated to mimic natural infection. To develop synthetic antigen vaccines it is necessary to understand how *Theileria* evades cattle immunity (Morzaria, Nene, Bishop & Musoke 2000). This evasion is thought to be mediated by continual generation of antigenically diverse strains during recombination in the tick (McKeever 2001). Cloning *Theileria* is a technique for studying the genetics of strain diversity. This communication is from a project which used transmissions with cloned *T. parva* to produce recombined stocks with hypothetical potential for immune evasion. We found abnormally low levels of infection in ticks with cloned *Theileria*. Therefore results of previous transmission experiments with uncloned *Theileria* of the same strains were compared with the performances of cloned *Theileria* in both cattle and tick infections in order to determine what levels of transmissibility can be expected when planning experiments.

Theileria parva of two strains which had been isolated from two sites in Kenya (Muguga and Marikébuni) were used. Both strains have been maintained as cryopreserved stabilates used for cattle to tick transmissions. The uncloned Muguga strain consists of a fairly homogeneous parasite population which can be distinguished by four genetic satellite markers (MS7, MS14, MS16 and MS25) developed by Oura, Odongo, Luguga, Spooner, Tait & Bishop (2003). The uncloned Marikébuni strain, however, is very diverse, and at least 48 out of the 60 markers described by Oura *et al.* (2003) reveal polymorphism between its constituents. Clones of these two strains were produced by infecting 1×10^7 peripheral blood mononuclear cells (PBMC) *in vitro* with a dose of *T. parva* stabilate equivalent to that carried by one infected tick. At 48 h after infection the cells were plated out by limiting dilution with non-infected PBMC in U bottom 96-well plates (Morzaria, Dolan, Norval, Bishop & Spooner 1995). The wells were scored for growth after 14 days and cells were harvested for DNA extraction and typing by satellite markers.

Male calves were Friesian at the Centre for Tropical Veterinary Medicine (CTVM) and Boran at the International Livestock Research Institute (ILRI) in Kenya. All were without previous exposure to *T. parva*. They were kept in isolation pens (20°C at CTVM) and monitored daily during infections for temperature, schizonts in the lymph node draining the site of infection and piroplasms in venous erythrocytes. The *Theileria* used as inocula were either stabilated sporozoites from infected adult ticks that had been partially fed for 4 days, or cell culture containing schizonts in PBMC, or in infected ticks. The doses of *Theileria* were calculated from previous *in vivo* and *in vitro* titrations to give patent piroplasm parasitaemias (0.1% or more of erythrocytes infected). Infections were controlled when necessary with tetracycline (short acting) injected intramuscularly at 10 mg/kg for up to 5 days starting from first pyrexia and appearance of schizonts. Experience has shown that this has no effect on infections in the ticks.

The ticks used were *R. appendiculatus* of a stock originating in the 1960s from Muguga in Kenya which have been maintained at CTVM since then. In addition, one experiment at ILRI used a stock designated as Muguga, of the same origin as above but maintained at ILRI since the 1970s. Transmissions were done by application of uninfected nymphal ticks to calves at a time corresponding with expected peak of piroplasm parasitaemia in red blood cells. Engorged nymphs detaching from the cattle were maintained at 28°C, 85% relative humidity for 28 days until completion of post moult development. Adults of both sexes in equal numbers (minimum sample of 32 ticks) that had detached on the day of peak piroplasm parasitaemia were assessed for infection with *T. parva* by incubation for 5 days at 37°C and 100% relative humidity, followed by dissection and staining of their salivary glands with methyl green and pyronin (Walker, McKellar, Bell & Brown 1979). Counts were made by microscopy of sporoblasts per pair of salivary glands. Data were not normally distributed thus analysis used Chi square, Mann Whitney U test and Kendall's rank correlation. Percentages were converted by arcsin and abundances of *Theileria* sporoblasts in ticks were transformed to $\log_{10}(n+1)$ to normalize the overdispersion of this variable (Büscher & Otim 1986). Two batches of uncloned and cloned *Theileria* in equal infection levels in ticks were compared for degree infectivity of the sporozoites for bovine cells after the *Theileria* were matured by partially feeding the ticks. Batches of ten ticks were surface sterilized and then ground in commercial medium using tissue grinders to release sporozoites. The suspensions of sporozoites were used to infect

cultures of bovine peripheral blood mononuclear cells in an infectivity titration (Wilkie, Kirvar & Brown 2002).

Historical data on transmissions using uncloned *Theileria* could not be standardized for use in the comparisons with cloned *Theileria* of this study. Three of the ten transmissions reported here representing cloned *Theileria* were of mixed infections using two different clones of *Theileria* sufficiently distinguishable by genetic markers for analysis of genetic recombinations. The outcome of these mixed infections was examined by polymerase chain reaction (PCR) using a sub-set of published satellite markers (Oura *et al.* 2003) which distinguish between the parent *Theileria* using their specified PCR conditions. The DNA used for this was extracted from lymph node and blood sampled repeatedly during the infections. From lymph node cells DNA was extracted using the Promega Wizard SV Genomic DNA Purification System and for blood samples the Qiagen QIAamp DNA Blood Mini Kit was used. The *Theileria* types in the ticks were also determined by PCR using the same satellite markers on DNA that was extracted either from dissected salivary glands or from whole ground ticks, with the same method as for lymph node cells.

The analysis of the mixed infections in Fig. 1 shows that the infections of animals BZ114 and BZ115 were

dominated by the *T. parva* Marikebuni clone and that the *T. parva* Muguga clone was barely detectable during the infection (Lanes 11 and 12). This was also seen in the infections in the ticks, where ticks fed on animal BZ114 did not pick up *T. parva* Muguga and for BZ115 Muguga alleles were only barely detectable (data not shown). We considered these mixed infections were more characteristic of infections from single sources and did not exclude them from further comparisons with single source infections.

Table 1 shows data from ten transmissions using cloned *T. parva* that met the criteria of producing patent infection with pyrexia at 40.0°C and piroplasms detectable by microscopy. These are compared with seven transmissions using uncloned *T. parva* meeting the same criteria. Sufficient data for a statistical comparison were obtained by combining results from infections with both strains of *T. parva*. Experience at CTVM and ILRI have shown that although these strains are different in immunological cross reactivity, they produce similar infections in cattle and ticks. Moreover, the five clinical variables measured in days were used to compare the Muguga and Marikebuni strains by Chi square tests between different pairs of calves. No significant differences were found between these pairs. The grouping of this data was considered justified for further comparison using Mann Whitney U test. The threshold for recording pyrexia was strict, at

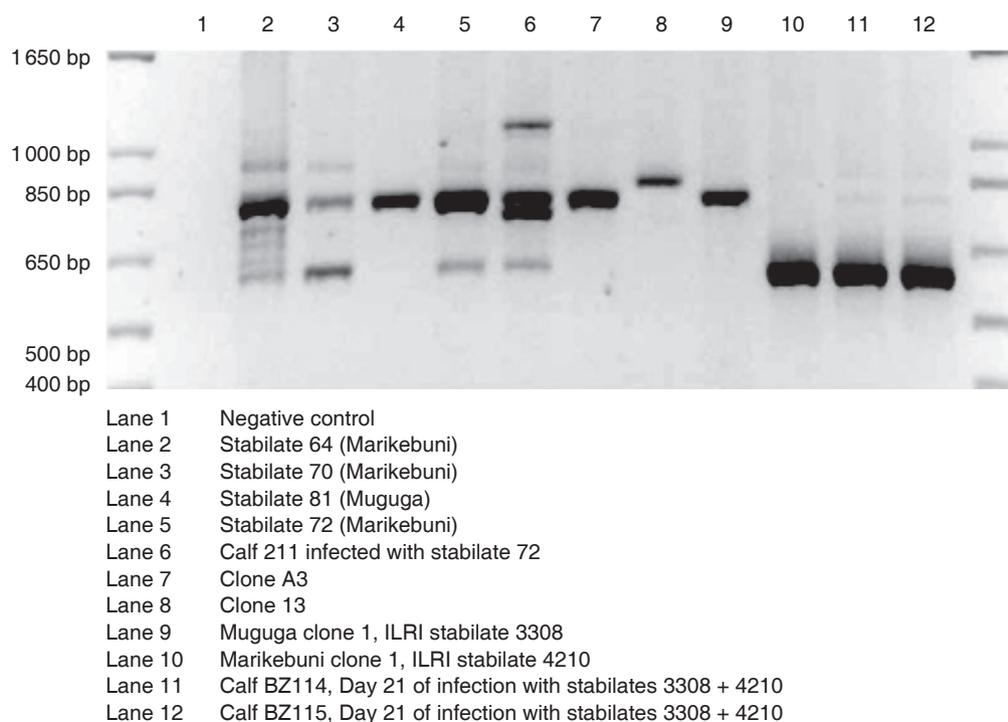


FIG. 1 Polymorphism in PCR size detected within the PIM gene of *T. parva*

TABLE 1 Infection characteristics of cloned compared to uncloned *T. parva* in cattle and Ticks. Ten transmissions with cloned *T. parva* and seven transmissions with uncloned *T. parva*, all meeting the criteria of patent disease with pyrexia and production of piroplasms. Analysis by Mann-Whitney U test

Strain of <i>T. parva</i>	Clone number	Infection method	Day 1 st pyrexia	Day 1 st schizont	Day 1 st piroplasm	Day max. piroplasm	Days pyrexia	Max. % piroplasms	Prevalence of sporoblasts per tick (%)	Abundance of sporoblasts per tick
Muguga	3308	Stabilate	8	10	12	17	11	5.6	60	17.9
Muguga	3308	Ticks	12	10	17	18	3	0.1	1.7	0.3
Marikebuni	3262	Ticks	9	11	15	17	9	1.2	35	4.5
Marikebuni	3262	Ticks	6	6	14	16	9	0.1	0	0
Marikebuni	13 + 17	cells	8	6	10	12	4	0.01	0.9	0.3
Marikebuni	4210	Stabilate	12	11	14	18	5	1.2	52	13.0
Marikebuni	4210	Stabilate	7	8	12	15	9	2.0	38	4.2
Marikebuni	A3	Cells	9	6	10	14	4	0.01	14	0.7
Muguga + Marikebuni	3308 + 4210	Stabilates	10	7	12	18	9	5.8	19	0.9
Muguga + Marikebuni	3308 + 4210	Stabilates	10	6	11	19	9	2.6	6	0.01
Medians			9.0	7.5	12.0	17.0	9.0	1.2	16.5	0.8
Muguga	Not	Stabilate	12	11	13	14	6	0.1	95	121
Muguga	Not	Stabilate	6	5	10	16	11	7.9	51	17.9
Muguga	Not	Stabilate	8	5	10	16	9	5.5	88	63.7
Muguga	Not	Stabilate	8	6	12	15	7	3.2	59	29.7
Muguga	Not	Stabilate	6	6	12	16	11	7.3	58	7.9
Marikebuni	Not	Stabilate	12	10	14	16	6	0.2	95	78
Marikebuni	Not	Stabilate	9	6	12	17	10	6.0	100	44
Medians			8.0	6.0	12.0	16.0	9.0	5.5	88.0	58.0
Probability			0.65	0.20	0.56	0.25	0.27	0.15	0.003*	0.002*

* Significantly different at $P < 0.01$

TABLE 2 *In vitro* assessment of infectivity of sporozoites of *T. parva* Marikebuni uncloned and clone 13. Data are mean numbers of wells with growth out of a total of 96 available wells for each concentration of cells per well

		9000 cells per well	3000 cells per well	1000 cells per well	300 cells per well	Total
4 day fed ticks	Uncloned	92	67	37	14	210
	Cloned	96	91	61	28	262
	Cloned	96	88	56	22	262
5 day fed ticks	Uncloned	96	91	80	38	305
	Cloned	93	71	29	14	207

40.0°C, and the number of days pyrexia was recorded up to Day 18. When ticks were used to infect, Day 0 of cattle infection was counted as Day 4 of tick feeding.

The course of infection of the calves using cloned *Theileria* was similar to that using uncloned *Theileria*. The median values of the characteristics measured in days were the same or representing milder disease in the cloned *Theileria* infections. The number of days pyrexia is the best single measure of these infections; it was 9.0 in both the infections with cloned *T. parva* and with uncloned *T. parva*. None of the comparisons of these characters between cloned and uncloned *T. parva* were significantly different. Although the level of piroplasm parasitaemia was more than twofold higher in the transmissions of uncloned *Theileria*, this difference was not significant. In contrast, the differences of infection with *Theileria* of the ticks from the transmission with cloned compared to uncloned *Theileria* were large and highly significant for both characteristics of prevalence of infection and abundance of infection. The relation between piroplasm parasitaemia and tick infection was examined using data from the uncloned *T. parva* transmissions. Kendall's rank correlations showed an inverse relationship, with negative and nearly significant correlations (piroplasms against prevalence -0.5 , $P = 0.06$; piroplasms against abundance -0.49 ; $P = 0.06$).

The results obtained at CTVM contrasted greatly with those from ILRI where, in totals of 29 transmissions with uncloned *T. parva* Muguga and separately *T. parva* Marikebuni, compared to 17 similar transmissions with cloned *T. parva* of both strains, the uncloned transmissions resulted in median tick infections having prevalence of 60.0% ticks infected and abundance of 20.8 sporoblasts per tick compared to cloned transmissions with prevalence of 72.0% and abundance of 22.0 (Paul Spooner, personal communication 2005). Table 2 shows that similar high levels of infectivity of sporozoites for bovine cells were found with the batches of cloned and uncloned

T. parva Marikebuni strain. There was no significant difference when compared by Chi square test.

It was important that only simple conclusions were drawn from the analysis because of the highly variable clinical course of infections with *Theileria*. We consider that we have found a characteristic of cloned *T. parva* that may have an influence on studies using clones and that this needs to be reported so that it can be incorporated into experimental procedures. Cloned *T. parva* produced a course of disease in calves with a clinical pattern without significant differences from that produced by uncloned *T. parva*. The cloned *T. parva* produced an acute infection that was potentially fatal and with sufficient piroplasms in the peripheral blood to expose feeding ticks to the typical massive numbers of the microgamete and macrogamete stage of *Theileria* in the tick's gut. Cloned *T. parva* infections in ticks were characterized by prevalences and abundances of infection that were both significantly lower than obtained with uncloned *T. parva*. It can be considered that this difference was due to the cloned *Theileria* infections producing a median of maximum piroplasm parasitaemias of 1.2% compared to 5.5% for the uncloned *Theileria*. However, these data are not significantly different and it is clear from Table 1 that low piroplasm parasitaemias can give rise to high prevalence and abundance of infection in some cases. Young, Dolan, Morzaria, Mwakima, Norval, Scott, Sherriff & Gettinby (1996) demonstrated, with transmissions in 113 calves of uncloned *T. parva* Muguga to *R. appendiculatus*, that there is a significant and positive linear regression of piroplasm parasitaemia with both prevalence and abundance of infection in ticks. However, this relationship was not simple; a large increase in piroplasm parasitaemia related to only a small increase in infections in the ticks. The data we present do not support a proposition that the difference in tick infections between cloned and uncloned *T. parva* was due directly to the difference in piroplasm parasitaemias. The infections of the ticks with cloned *T. parva* in the tick's salivary glands

matured normally to produce sporozoites of infectivity to cattle cells that were similar to uncloned *T. parva*.

These results indicate that there was a characteristic of cloned *T. parva* that either reduced the rate of syngamy or viability of the zygotes and kinetes in the tick. It may be important that the meiotic reduction division of *T. parva* is thought to occur in the kinete stage (Gauer *et al.* 1995). The tick gut and haemolymph between the gut and salivary glands are potentially hostile from the likely effects of an innate immune system of this arthropod. *Theileria parva* in high levels of infection in *R. appendiculatus* can harm the salivary glands and thus it is likely that this species of tick has evolved immune defences against it (Watt & Walker 2000). Genetic experiments using cloned *Theileria* should be adjusted to this anomaly.

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